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BIOLUMINESCENCE

By

**E. NEWTON HARVEY, RUBERT S. ANDERSON, JOHN B. BUCK, AURIN M.
CHASE, HENRY EYRING, AND FRANK H. JOHNSON**



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* This series of papers is the result of a Conference on Bioluminescence, held by the Section of Biology of The New York Academy of Sciences, November 8, 1946.

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THE NEW YORK ACADEMY OF SCIENCES

INTRODUCTORY REMARKS: A GENERAL SURVEY OF BIOLUMINESCENCE

By E. NEWTON HARVEY

Osborn Professor of Biology, Princeton University, Princeton, New Jersey

Bioluminescence occupies a somewhat paradoxical position among biological subjects. It is not only a specialized and restricted field of inquiry but at the same time a very broad one. It is restricted in that relatively few of the very large number of animal or plant groups have developed the singular ability to produce light, and broad in that it presents problems of the greatest interest to every branch of biology—to the systematist, histologist, morphologist, physiologist, biochemist, and ecologist as well as to the student of animal behavior and of evolution. Furthermore, the beauty and the mystery of animal light have always aroused the interest of the traveler and the student of other fields such as pure chemistry and physics.

Among the great chemists and physicists of the past who have taken a more than casual interest in bioluminescence are Boyle, Newton, Franklin, Priestly, Réaumur, Dessaignes, Becquerel, Davy, and Faraday. Many naturalists have been fascinated by luminous organisms, and it is rather surprising to find that Darwin, despite the variety of his travels and the breadth of his interest, casually mentions luminous animals and draws no inferences for the theory of natural selection from the widespread ability of many living forms to emit a cold light. Among biologists who have made a special study of animal light may be mentioned the names of Anderson, Beijerinck, Buck, Chase, Dahlgren, Dubois, Ehrenberg, Eyring, Giese, Harvey, Heinrich, Heller, Johnson, Kanda, Kishitani, Kluyver, Krukenberg, Mangold, McDermott, McElroy, Okada, Panceri, Phipson, Pierantoni, Pratje, Quatrefages, Spallanzani, Tilesius, Trojan, van der Burg, van der Kirk, van Schouwenburg, and Zirpolo. We regret that more of our contemporaries in this group were not present at the conference.

About 40 different orders of animals are self-luminous. These include such diverse organisms as bacteria, at least two groups of fungi, radiolaria, dinoflagellates and cystoflagellates; sponges, hydroids, medusae, siphonophores, pennatulids, ctenophores, and nemerteans; 7 families of marine worms, earthworms; ostracod, copepod, decapod and schizopod crustaceans; myriapods, possibly spiders; spring-tails, flies, and beetles; brittle-stars, bivalves, nudibranchs, and two orders of squid; balanoglossids, ascidians, and several orders of fish, both elasmobranchs and teleosts. The phyla Platyhelminthes, Nemathelminthes, Trochelminthes and all vertebrates above the fish (amphibians, reptiles, birds, and mammals) are non-

luminous. All higher plants (Bryophytes, Pteridophytes, and Spermatophytes) also contain no luminous forms.

Among the luminous groups listed above, only terrestrial or marine animals produce light, while fresh-water organisms do not, even though closely related to luminous marine species. The nearest approach to a luminous fresh-water animal is an aquatic firefly larva breathing by

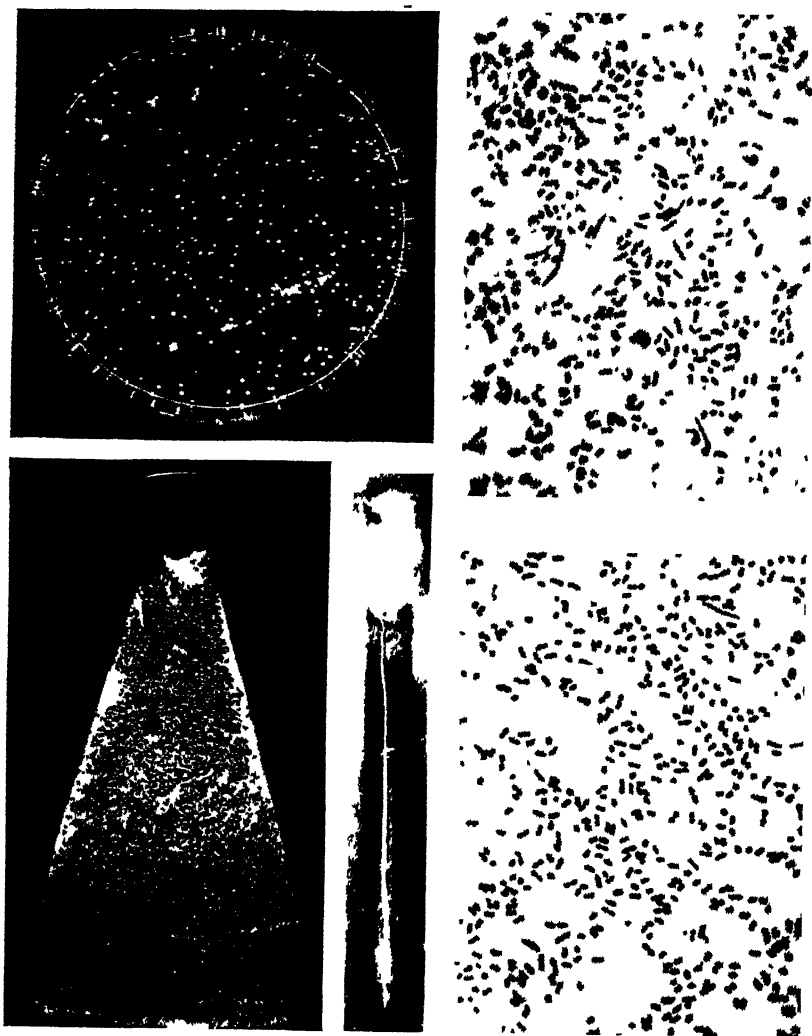


FIGURE 1. Left, luminous bacterial colonies growing on a Petri dish, flask, and test tube of culture medium (after Molish). Right, luminous bacteria highly magnified, *Pseudomonas toayamensis* (above) and *Coccobacillus ikienis* (below), after Kishitani.

tracheal gills. The diverse and apparently chance distribution of luminescence in the living world suggests that the mechanism for producing light has developed in the course of evolution from some one of the chemical systems already generally present in cells and probably from one concerned with cell respiration.

Hence, much is to be gained from a study of luminescence in organisms such as luminous bacteria, where the cell respiration is intimately connected with the production of light. These bacteria occur in the sea and frequently form colonies on dead fish or squid and also on meat in refrigerators. They are of several species, are non-pathogenic to humans, and easy to culture on 2 per cent peptone, 1 per cent glycerine sea water agar at room temperature or below. A pinch of CaCO_3 can be added to maintain the proper pH. A good idea of their appearance can be obtained from FIGURE 1.*

In addition to such saprophytic bacteria, there are also parasitic forms. Various organisms, such as sand fleas, shrimps, midges, or caterpillars may become spontaneously infected with parasitic luminous bacteria and develop a luminous malady that is finally fatal. In the meantime, the host animals move about and would be mistaken for true luminous organisms if the origin of the light were not known.

Finally, certain squid and fish may be occasionally or regularly luminous because of the harboring of luminous bacteria in their glands. No harm results to the host from these bacteria. In the remarkable cases of the East Indian fish, *Photoblepharon* and *Anomalops* (shown in FIGURE 2), a special light organ has been developed under the eye in which luminous bacteria are always present. They live between special long cylindrical cells which are richly supplied with blood capillaries. Moreover, the continuous light emission of the bacteria can be shut off by a screening mechanism of the fish. In *Photoblepharon*, there is a fold of black membrane, like an eyelid, that can be drawn up over the organ, thus obscuring the light. In *Anomalops*, the light organ is attached at the anterodorsal corner by a hinge that allows the whole organ to be turned over and downward into a black-pigmented groove or pocket, so that none of the light-emitting surface is visible.

These two fish alone present enough problems for the evolutionists. Why should these two very closely related genera have developed two totally different methods of shutting off the light? When do the bacteria get into the organ? Nothing is known about the embryology of the fish or why so large a light surface should appear immediately under the eye. Such problems might warrant an expedition to the Banda Sea, the only

* FIGURES 1-4 are reproduced from *Living Light* by E. Newton Harvey, courtesy of Princeton University Press.

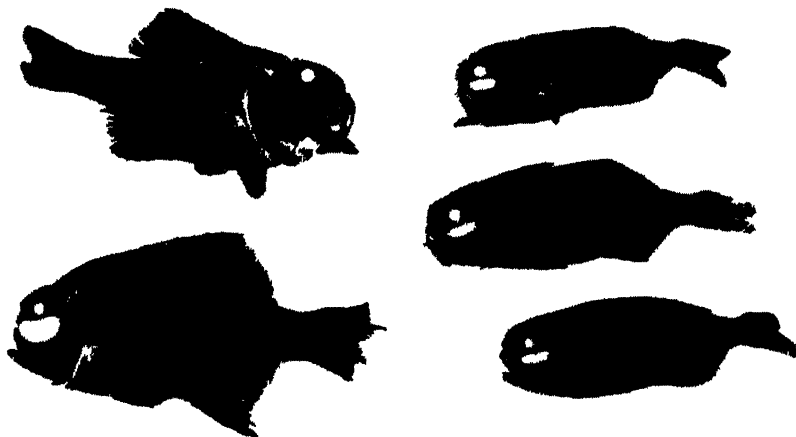
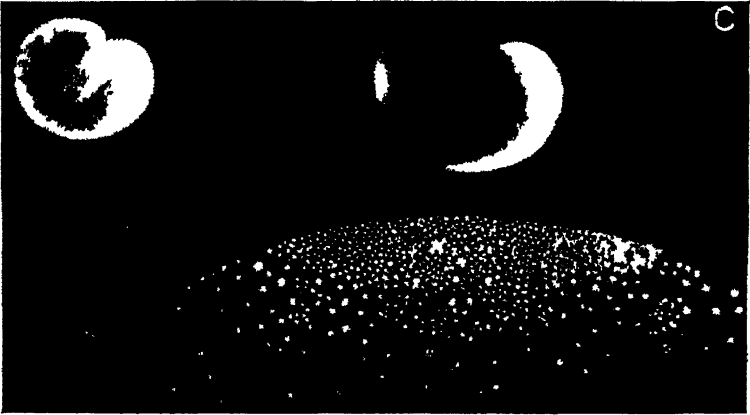
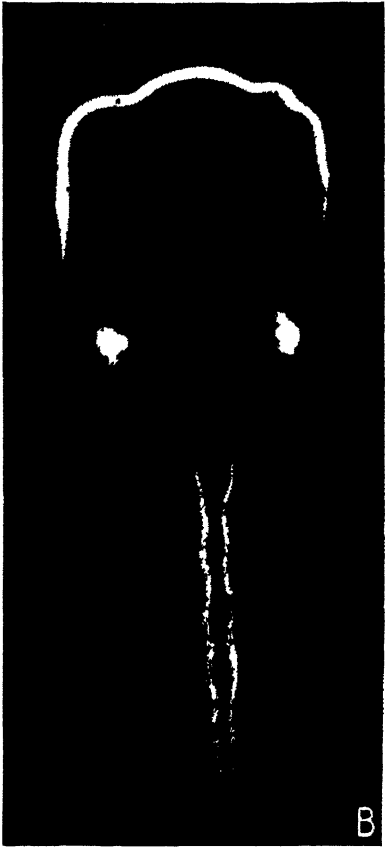
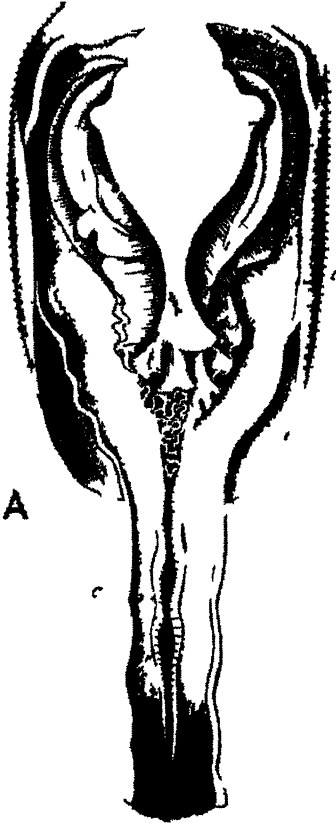


FIGURE 2 Above the fish *Photoblepharon palpebratus* swimming in water of the Banda Islands at night (After Dahlgren from a painting by Bruce Horsfall) Below *Photoblepharon palpebratus* (left) and *Anomalops latoptron* (right) photographed from dead specimens showing the large luminous organ under each eye whose light comes from symbiotic luminous bacteria

(See opposite page)

FIGURE 3 A *Phola dactyla* the luminous clam with which Raphael Dubois demonstrated the luciferin-luciferase reaction in daylight B The same at night to show the luminous regions (after Panceri) C The flagellate *Noctiluca scintillans* showing luminescence of two whole organisms and the appearance under the microscope where the light can be seen to come from small discrete luminous granules (after Quasthoff)



place in the world where *Photoblepharon* and *Anomalops* occur in considerable numbers

Bioluminescence is actually a chemiluminescence, *i e*, light production during a chemical reaction. It is quite fitting, therefore, that a conference on bioluminescence should begin with light production by pure organic substances in solution. Many such compounds are known and the light emitted by some is indeed brilliant. Examples of these chemiluminescences will be discussed by Dr. Anderson.

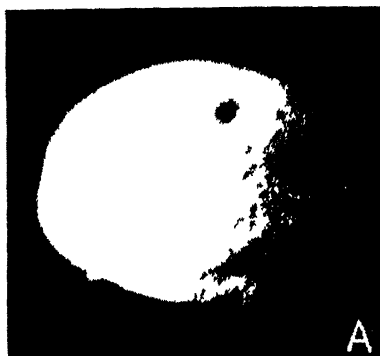
The chemiluminescences responsible for the light of organisms involve the oxidation of a compound, luciferin, in presence of an enzyme, luciferase. In some organisms (fireflies and bacteria) the oxidation is intracellular, in others (*Cypridina* and *Pholas*) extracellular, a large amount of luminous secretion being stored in a special gland. In certain organisms (bacteria, protozoa), a single cell may produce the light. In others (shrimp, squid, fish), accessory structures have been developed, so that the light organ can be truly described as a lantern, with lens, reflector, and sometimes both color and opaque screen. In the bacteria and fungi, the light emission is continuous day and night; whereas in all other forms, it appears only on stimulation.

Success in the study of any biological phenomenon is dependent on particularly favorable experimental material, *i e*, what we have come to speak of as classic forms. A good example of classic organisms for study are the luminous bacteria which have already been mentioned. Their contribution to a study of bioluminescence will be discussed by Dr. Johnson. Unfortunately, no one has as yet succeeded in extracting luciferin and luciferase from luminous bacteria. These substances, first demonstrated by Raphael Dubois in the elaterid beetle, *Pyrophorus*, in 1886, and later obtained from the mollusc, *Pholas dactylus*, and studied in detail by Dubois, are best extracted from luminous animals with extracellular luminescence, where a large quantity of luminous secretion is formed. *Pholas* is pictured in FIGURE 3A and B.

In 1916, during a trip to Japan for the collection of luminous squid I noticed the abundant luminous secretion of the small ostracod crustacean, *Cypridina hilgendorfi*, and was at once able to establish the presence of luciferin and luciferase. These substances are secreted from separate long, single, gland cells opening at pores near the mouth. Moreover

(See opposite page)

FIGURE 4. A. A single ostracod crustacean, *Cypridina hilgendorfi*, enlarged showing the black eye-spot tip of swimming legs (below) and protuberances of luminous gland (right). B. Dried *Cypridinae* life-size photographed on a cloth. C. A photo-cell and string galvanometer record of light intensity (vertical) as a function of time (horizontal), when *Cypridina* luciferin and luciferase are mixed. D. A similar record of ink mixed with water to determine time of mixing. Light intensity in arbitrary units. Each large division represents 0.2 second. (After Harvey and Snell). E. A cross-section of the gland of *Cypridina*. F. A longitudinal section of the gland region showing two types of single gland cells opening by separate pores and cross-striated muscle fibers, whose contraction squeezes the secretion into the sea water. (after Yatsu)



A



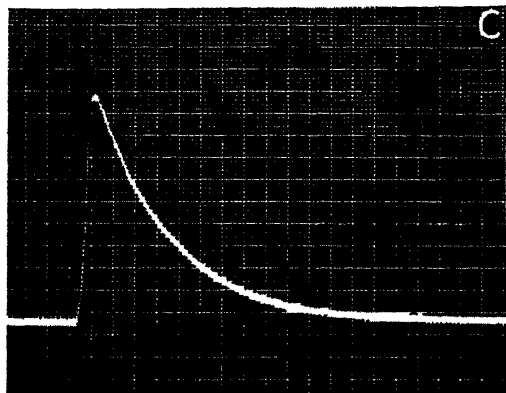
B



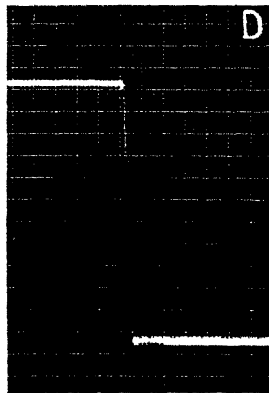
F



E



C



D

the whole animal, less than an eighth of an inch long, can be obtained in fair quantities and, when dried rapidly, indefinitely retains the ability to luminesce whenever moistened. Such dried material has been invaluable in a study of the chemistry of luciferin, which, together with some observations on *Cypridina* luciferase, will be presented by Dr. Chase. *Cypridina*, shown in FIGURE 4A and B, has become the classic organism for chemical study of bioluminescence. The histology of the gland is pictured in FIGURE 4E and F, and a record of *Cypridina* luminescence in FIGURE 4C and D.

One of the almost universal characteristics of luminous forms (except for the bacteria and fungi) is the ability to flash on stimulation. The effect is particularly well seen in the "phosphorescence of the sea", where waves dashing on shore, the splash of oars, or the wake of a boat are outlined by the flash of thousands of small marine organisms, mostly flagellates, stimulated mechanically by the motion of the water. *Noctiluca*, the best known of the flagellates, is shown in FIGURE 3C.

Higher organisms, also, possess a mechanism for turning their light on and off, well seen in the firefly flash. In fact, fireflies are classic material, not only for study of the physiological problem of flashing, but also for investigation of the fine histological details of luminous organ structure, which is necessary for an understanding of the physiology of flashing. Dr. Buck will consider these aspects of the bioluminescence problem and will also, I hope, describe his ecological studies on the firefly and the use of the flash as a mating signal. We need a scientific explanation of the use of the light in many luminous animals as well as of the mechanism of cold light emission itself, that unique phenomenon which, in the form of the fluorescent lamp, is now used to light our homes.

CHEMILUMINESCENCE IN AQUEOUS SOLUTIONS

By R. S. ANDERSON

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In trying to analyze a biological process such as bioluminescence, a beginning is often made by relating the biological observations to the most similar parts of pure chemistry and physics. This has been done in connection with light given off by living organisms or by extracts from them, and at least the most common type of luminescence has been generally agreed upon. In the case of the best known system, *Cypridina* luciferin and luciferase, the light is clearly the result of a chemiluminescence. Even the reaction producing it, an oxidation by oxygen, is a common type of reaction producing chemiluminescence. The bioluminescence of many forms seems to be similar, in various ways, to that of *Cypridina*, and these forms are therefore believed to contain chemiluminescent systems even though they may never have been separated from the cell.

It would be helpful if there existed a well developed body of information about the chemiluminescence of known compounds in solution. Unfortunately this is not the case, and answers to most of the questions which might arise in the study of bioluminescence cannot be given. It is the purpose of the present review to collect a number of the many scattered observations made, particularly in recent years, on chemiluminescence in water solutions, with little reference to the biological systems.

From early times, the characteristic of bioluminescence and chemiluminescence which has seemed most striking has been light-emission at ordinary temperatures, in contrast to its usual association with high temperatures. This property is also shown by fluorescence. The actual emission process is probably analogous. It results, most immediately, from loss of a quantum of energy during the change of an excited electronic state to the ground state or some other state having less energy than the initial excited one. The major difference between chemiluminescence and fluorescence is, then, the means by which the excited electronic state is first produced. In a chemiluminescent reaction, a part of the energy resulting from the reaction is channeled in a very particular way instead of being dissipated as heat. In fluorescence also, the energy is introduced into the molecule in a specific way, *i.e.*, by the absorption of radiation. Neither the emission of the radiation, nor the production of the excited state, need depend on a high temperature.

Even though excited states have been produced, in the one case by a chemical reaction and in the other by absorption of radiation, the excess

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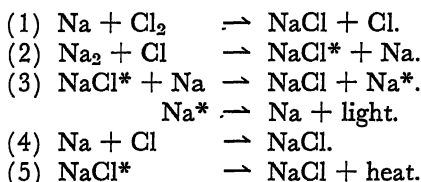
energy need not be lost as radiation. While some of the molecules having the excess energy may emit light, others may lose it as heat or in chemical reaction. The ratio of the number of light emitters to those experiencing energy loss in other ways can be modified by various means, particularly by the addition of so-called quenching agents, which decrease the amount of light. Quantitatively, light emission at ordinary temperatures is a relatively uncommon process. For instance, absorption of light, capable of raising electrons to excited states, occurs at least in all colored solutions. Yet many colored solutions are not fluorescent at all and, of those that are, the efficiency rarely approaches one.

The original production of the excited state from a chemical reaction is supposed to result from a crossing or near approach of potential energy surfaces. Eyring *et al.*¹ also consider that it may occur as a result of direct excitation by black body radiation, at least in the case of some extremely faint instances of chemiluminescence studied by Audubert.² The crossing or near approach of potential energy surfaces may perhaps be visualized in some way such as the following. If, for a compound, some configuration of the nuclei exists, a distance in the diatomic case in which the system has approximately the same potential energy with its electrons in the ground state and in an excited state, a shift may occur from the ground state to the excited state. The system may be brought to this critical configuration by a chemical reaction. When the configuration changes, the electron excitation energy is trapped and may then be lost as a quantum of light.

Considering, now, specific systems, it was stated above that no systems in solution are well understood. In fact, until fifteen or twenty years ago no reactions of specific compounds in aqueous or other solvents were known which gave off as much light for comparable amounts of material as the extracts from organisms. Lophine and the Grignard compounds probably approached them most closely. That is no longer true, since the description of the bright chemiluminescence of aminophthalhydrazide by Albrecht in 1928³ and of dimethylbiacridinium nitrate by Gleu and Petsch in 1935.⁴

To find a chemiluminescent reaction which can be described with some completeness, it is necessary to turn to a much simpler system, a gas reaction. Gas reactions seem remote from bioluminescence or even chemiluminescence in solution, but they may act as a guide in a number of ways, and some of the same general experiments apply. In 1928 Polanyi and his co-workers reported extensive experimental work on the reaction of the alkali metals with several halogens and halides. For our purpose, it is necessary to consider only one of these reactions, that between sodium vapor and chlorine.⁵ When these two elements react under the proper conditions, light is emitted. The sodium vapor is introduced at one end of a tube at a pressure of only 0.001 or 0.01 mm. Hg. The chlorine is

introduced into the tube as a jet. The simplest condition is to have the sodium in excess. The reaction occurs where the two gases meet. From the character of the emitted light, the effect of pressure of reactants, the effect of foreign gases, and from the position of the luminescence or flame with respect to the deposit of reaction product (NaCl), the several steps of the reaction have been obtained. The following major events are believed to occur:



Most of the light which is emitted has been identified as the D-line of sodium. This immediately shows the emitting material to be primarily sodium atoms. The frequency of the D-line is equivalent to 48.5 Cals. Reaction 1 furnishes only 35 Cals., which is not enough to produce the light emitted. Reaction 2, giving 75 Cals., provides more than the necessary energy for the emitted light and is believed to be its source. That the sodium atom produced in reaction 2 is not excited as it is produced, follows from a number of additional facts. The chemiluminescence of the reaction is quenched by foreign gases more strongly than the resonance emission of the D-line of sodium. This means that the excess energy is retained long enough for collisions to occur and dissipate the energy in some other way. The emission of the D-line by excited sodium occurs after about 10^{-8} seconds. It is, therefore, suggested by Polanyi that the excess energy is retained by some other material for a longer period than 10^{-8} seconds. This material is believed to be the sodium chloride. The excess energy of the sodium chloride molecules is then supposedly transferred to sodium atoms during collisions as indicated in reaction 3. The excited sodium atom thereupon loses the energy as light. Reactions 4 and 5 are competing non-luminescent reactions occurring at appreciable rates only at the wall. The very large effect of changes in the pressure of sodium vapor results from differential effects on the several reactions; that is, increased pressure of sodium favors reactions 2 and 3 as compared to 4 and 5, and an increased yield of light results. An increase of sodium from 0.001 to 0.01 mm. Hg. pressure increases the quantum yield, per two atoms of sodium reacting, from about 1 per cent to about 35 per cent. The calculated maximum quantum efficiency with still greater pressures of sodium was set at about 0.85, although this was not realized experimentally.

A fairly complete description, therefore, can be given of the chemical reactions leading up to the emission of the light and the side reactions

which compete with those responsible for chemiluminescence. Even in this apparently very simple case, quite a number of complicating factors are present. In view of these complications in a simple gas reaction, it is not surprising that the description of events in the reaction of a large organic molecule in solution is so inadequate. However, although some of the features of these gas reactions are special to them, the general experimental approach through spectral emission curves, concentrations of reactants in relation to light emitted, quenching, etc., are applicable to studies in solution.

The number of known chemiluminescent reactions in solution is large, although this number is a very small fraction of the total known reactions. Harvey, in his book⁶ indicates many of these reactions. Oxidation is a usual type of reaction, and pyrogallol, lophine, and the Grignard compounds are some of the better known materials undergoing reaction. A number of common systems previously considered to be non-radiating have been studied by Audubert.² By the use of specially designed counters, he reports finding very low intensities of ultraviolet light associated with many reactions.

This discussion will omit those reactions which show a very low efficiency and consider the two which seem most comparable, in amount of light emitted, to the gas reaction and to the bioluminescences. This distinction is, of course, arbitrary to some extent, since an intense chemiluminescence or fluorescence may be extinguished by a change of conditions. Recognizing this, it still seems that for some experimental purposes more emphasis should be placed on the quantitative differences between various reactions under the most favorable conditions known. In most of the known systems, the actual fraction of the total number of reacting molecules which give off light is extremely small. The fact that they have been observed at all is due in many examples to the extreme sensitivity of the eye as a detecting instrument. It may not be a case even of one in thousands or tens of thousands, but one in millions of molecules. In the reactions studied by Audubert, only one in 10^{14} or 10^{15} of the molecules which reacted emitted radiation. The aminophthalhydrazide³ and the dimethylbiacridinium compounds⁴ mentioned earlier are, in aqueous solutions, the most efficient light-producing systems which have so far been discovered. Each will be considered in some detail. Although a considerable study has been made of these reactions, definitive results upon which all authors are in agreement have not appeared.

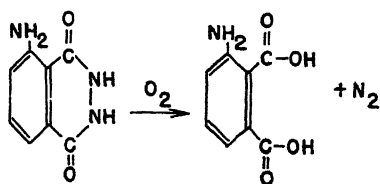
The Phthalcyclohydrazides

Albrecht,³ in 1928, studied extensively the chemiluminescence of several phthalcyclohydrazides of which the brightest and most studied was 3-aminophthalcyclohydrazide.* This hydrazide emits a blue light when

* Also called luminol and, by Chemical Abstracts, 5-amino-2,3-dihydro-1,4-phthalazinedione.

it is oxidized in alkaline solution with a number of oxidizing agents such as hydrogen peroxide, potassium ferricyanide, or sodium hypochlorite. Convenient methods of preparing the hydrazide for demonstrating the reaction⁷ and additional studies of related compounds⁸ appeared a few years after Albrecht's work.

Albrecht formulated the overall reaction as:



The time course of the reaction is markedly dependent upon the conditions and especially upon the oxidizing agent which is used. Albrecht⁸ found that, with most oxidizing agents, the light is dim and of short duration. With hydrogen peroxide, the reaction lasts for a much longer time although the light intensity is low. The intensity of the light can be much increased, although the duration is decreased, by having present in addition to the hydrogen peroxide, ferricyanide, hypochlorite, or one of a number of compounds which appear to act catalytically. In general, hydrogen peroxide in the medium is necessary for the large yields of light. Other authors^{6, 10-20} have subsequently studied a variety of catalysts. Manganese dioxide, colloidal platinum, hemin or hemoglobin, many metal complexes, and ozone are a few of the materials which produce increased light intensity. Weber and his collaborators more recently have studied the relation of other trivalent iron complexes,²¹ copper complexes,²² RuCl₃ and VOSO₄,²³ to the chemiluminescent reaction. Whether the compound is to be looked upon as a reactant or a catalyst depends, according to these authors, upon its rate of reoxidation by atmospheric oxygen.²¹

The form of the relation between intensity of light and time depends on the catalyst used. For instance, Weber *et al.*²¹ consider that the light intensity decreases exponentially with time with a number of iron catalysts. Stross and Branch,²⁴ using hydrogen peroxide and ferricyanide, find the curve of log intensity against time to be convex upward. They also observe that the initial point at 0.2 seconds lies below the smooth curve and suggest that the luminescent reaction may not be the initial one. The intensity-time relation of the uncatalyzed, and hence slow, reaction of hydrogen peroxide with the hydrazide depends on the concentration of hydrogen peroxide and alkali.^{25, 26} Many such curves show a point of inflection because the intensity of luminescence does not decrease as rapidly during the initial portion of the reaction as it does later.

It has long been known^{3, 10} that the hydrazide would show visible luminescence at extremely great dilutions, one part in 10^8 parts of water. The quantitative efficiency of the light emission in terms of amount of light per mole of hydrazide present has also been studied. Harris and Parker,²⁷ by means of a flowing system, studied the light emitted by the hydrazide in a medium of 0.35 M sodium hydroxide, hydrogen peroxide, and sodium hypochlorite. The reaction was so fast that it was confined to a spot of light even in the flowing system. They found the efficiency to be markedly sensitive to the concentration of hydrogen peroxide, with the best condition approximately 4 moles of hydrogen peroxide per mole of hydrazide. Under their optimum conditions, from 0.003 to 0.005 as many quanta of light were produced as there were hydrazide molecules present, assuming that all of the light was emitted at 4250 Å. Use of potassium ferricyanide instead of the hypochlorite gave a more diffuse spot of light, presumably because the reaction rate was slower.

Stross and Branch²⁴ later published a study of the hydrazide also in a flowing system. They used varying concentrations of sodium hydroxide, hydrogen peroxide, and potassium ferricyanide. Instead of studying the light emission from a spot, they measured the light intensity at varying distances from the point of mixing. The maximum number of quanta observed per molecule of hydrazide, assuming that the light was emitted at 4570 Å, was 0.36. This is 50–100 times as great as the value given by Harris and Parker. Stross and Branch do not refer to the earlier work, so that no explanation of the discrepancy is available, apart from the different oxidizing agents.

The emitted light covers a broad region of wavelengths with a suggestion of a double maximum.^{3, 28} The precise location of the curve is variously reported and may depend on the oxidizing agent or catalyst used with hydrogen peroxide. For instance, Eymers and Van Schouwenberg²⁸ report the maximum at 4400–4600 Å with hemin, while with ferricyanide the emission extends farther into the blue.

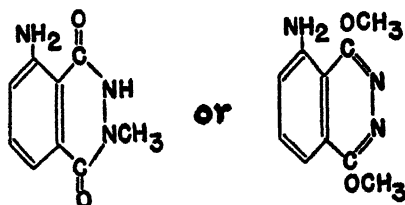
The emitted light, unlike the gas reaction of sodium and chlorine, gives no immediate clue as to the identity of the emitting molecule. An attempt has been made, however, to obtain some information by comparing the chemiluminescence and the fluorescence of the hydrazide and other compounds. When the fluorescence emission curve of a compound is similar to the chemiluminescence emission curve, it has been considered as evidence supporting the idea that this same compound also emits the light in the chemiluminescent reaction. Although it is not certain that the same molecule producing the two types of luminescence would necessarily emit identical spectral distribution curves, it has been considered a plausible assumption with which to begin. Such a comparison was first made by Albrecht.³ An immediate complicating factor appears for the hydrazide. The chemiluminescence occurs best in alkali-

line solution, while the fluorescence appears in neutral or acid solution. Albrecht considered the two spectral distribution curves to be similar, even though the maxima were about 200 Å apart. This difference he ascribed to the different pH's of the solutions. His view was supported, in part, by Sveshnikov,²⁵ who brought the maxima closer together by bringing the pH's of the chemiluminescent and fluorescent solutions together more closely. The comparison of fluorescence and chemiluminescence of the hydrazide has been studied also by Eymers and Van Schouwenberg,²⁸ who believe that the two curves are essentially different. A comparison has also been made by Albrecht of the chemiluminescence and fluorescence of other phthalcyclohydrazides. He stated that the color of the emitted light shifted in a parallel manner in a series of derivatives. Whatever view may be the correct one, most discussions of the actual chemiluminescent reactions assume that the emitting molecule is the initial hydrazide itself or some closely related compound.

It is a matter of practical and perhaps of theoretical importance to know if the emission curves, as observed experimentally, represent the emitting molecule. Distortion could occur by the presence of compounds which either absorb some of the emitted light or fluoresce under its influence. According to the published curves,^{25, 27, 29, 30} absorption by the hydrazide itself should not greatly influence the emission in the visible region, although the same may not be true for other reactants and catalysts which have been used. That fluorescence of various dyes occurs when they are dissolved in a solution of the hydrazide emitting chemiluminescence, has been shown by a number of authors.³¹⁻³³ Also, mixtures of hydrazide with dimethylbiacridinium salt, in solutions where only the hydrazide would show chemiluminescence, give the green light of the acridinium chemiluminescence or fluorescence.³⁴ Tamamusi³⁵ has favored the idea that such light emissions may not be true fluorescences but actual transfers of energy by collision of hydrazide with dye followed by light emission from the dye. A transfer of energy during collision of sodium chloride and atomic sodium is included in the gas reaction described above. However, no similar process has been shown for chemiluminescence in solution and, according to Weber and Ochsenfeld,³⁶ the experimental results on hydrazide can be quantitatively explained without assuming such an energy transfer.

The relation of structure to the luminescence has been clarified by work from a number of laboratories.^{3, 9, 10, 37-45} Several conclusions were summarized by Drew⁴¹ in the 1939 Faraday Symposium on luminescence, although agreement is not complete.^{42, 43} According to Drew, the 6-membered ring hydrazide is an essential requirement for chemiluminescence. Neither the open chain nor the 5-membered ring hydrazides show any luminescence. Apparent exceptions to this rule are explained as due either to a preliminary change into the 6-membered ring

compound or to the presence of traces of it as an impurity. It has also been found^{9, 39, 40} that methyl group substitutions such as



give non-luminescent compounds. Therefore, it is concluded that both hydrogens must be present on the nitrogen atoms or as the di-enol. The sensitivity of the eye to light requires exceptionally rigorous purification of the compounds studied to avoid misinterpreting the data. Drew and Garwood⁴⁰ used as many as eight recrystallizations to free some materials of traces of chemiluminescent impurities. More recently, Huntress and Gladding⁴⁵ have shown that changing the order of the carbon atoms and nitrogen atoms in the ring in the several possible ureas and quinoxalines, results in compounds which do not luminesce although they appear to oxidize.

Given the 6-membered ring hydrazide, Albrecht⁸ showed in his thesis that substituents in the other ring greatly influenced the amount of light emitted. An amino group in the 3 or 4 position increased the amount of light as compared with the unsubstituted phthalhydrazide. Position 3 was more effective than 4. The nitro group decreased the amount of light. Drew⁴¹ concluded that, in general, substitutions in the *ortho* positions with respect to the cyclohydrazide ring had greater quantitative effects than the corresponding substitutions in the *meta* positions. The nature of the substituent in the benzene ring has a great effect on the intensity of the light emitted and also influences its color.^{8, 38} Some relative intensity measurements have been made^{38, 40, 42} which permit a listing of substituent groups in the approximate order of light emitted. Groups such as NH_2 , NHMe , OH , $\text{NH}\cdot\text{NH}_2$, in that order, and to a lesser extent chlorine, bromine, and iodine, increase the amount of light emitted as compared to the unsubstituted compound. On the other hand, groups such as NO_2 increase the light very little or decrease it. In general, Drew and Pearman³⁸ consider the first class to correspond to the *ortho* and *para* directing groups of organic chemistry, and the second to *meta* directing groups.

It may be that this apparent relation between the amount of chemiluminescence and the directive influence of substituent groups comes about through the usual effect of the groups on the chemical reaction. In this connection, however, some results obtained by West⁴⁶ on the

fluorescence of naphthalene and its derivatives are of interest. The following relative efficiencies of fluorescence are reported:

NH ₂	:	1
β -OH	:	0.75
β -CN	:	0.5
α -COOH	:	0.3
Naphthalene	:	0.15
α -chlor	:	0.05
NO ₂	:	none.

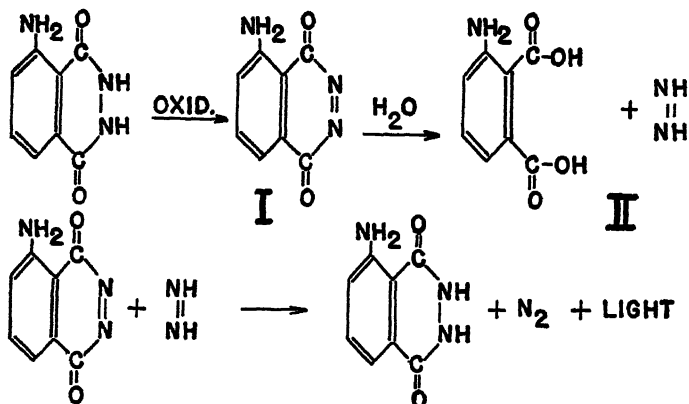
Although these results presumably do not involve a chemical reaction, the various groups again have a striking and specific effect on the quantitative result. Also, the order of the groups is approximately the same as for the chemiluminescence, with the exception of cyanide and carboxyl which are not given by Drew and Pearman.³⁸ These two are *meta* orienting groups.

Compounds present in solution also have a marked influence on the amount of chemiluminescence, just as in fluorescence. For instance, it has been found that traces of hydroquinone^{24, 42} cause a great diminution in the light intensity. A series of papers by Weber and his collaborators have reported studies on the influence of many compounds and the halide ions on the fluorescence⁴⁷ and chemiluminescence²¹⁻²³ of the hydrazide. The influence on chemiluminescence may operate both through the chemical reaction and through quenching, which is analogous to fluorescence quenching. That the effect of chloride and bromide ions on a chemiluminescent reaction need not be to decrease but may increase the amount of light emission, has been shown with the *Cypridina* luciferin and luciferase system.⁴⁸

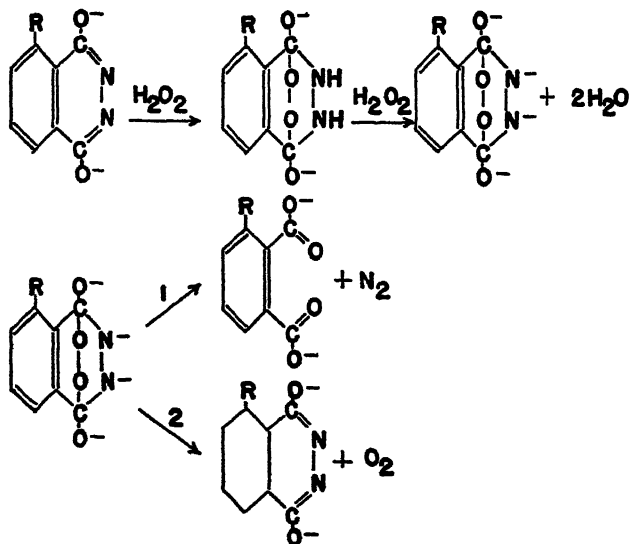
The actual reactions which produce the chemiluminescence are not agreed upon, although several authors^{3, 21, 24, 41, 49} have suggested systems which account for a certain amount of the experimental data. Albrecht's³ original formulation (see following page) has not been accepted.⁴¹ The azodiacyl compound (I) is unknown, and the diimine (II) is hypothetical. However, in 1942, the finding of a compound, colored and unstable, believed to be (I) was reported by Kautsky and Kaiser.⁵⁰ A solution of this material, free of oxidizing agent or oxygen, is said to emit light when made alkaline. No further work on its isolation and identification has appeared.

Meanwhile, Drew and Garwood⁴⁰ reported the isolation of a sodium salt of the peroxide of the 3-aminophthalcyclohydrazide. This compound, dissolved in water, gives off luminescence when hemoglobin is added to the solution. The intensity increases when alkali is added.

The same authors also report that the extent of the destruction of the hydrazide during chemiluminescence depends on the conditions, includ-



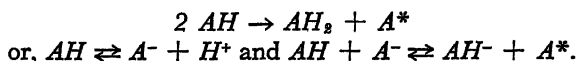
ing particularly the oxidizing agent used. Destruction, with evolution of all of the hydrazide and much of the amino nitrogen, occurs with hypochlorite and permanganate. However, when to a solution of hydrazide and hydrogen peroxide, sodium hypochlorite was added slowly, as little as one-seventh of the hydrazide nitrogen was liberated. The authors suggest, therefore, that most of the hydrazide can be recovered unchanged and that the destruction of this ring may be a side reaction and not an integral part of the chemiluminescent reaction. Their provisional formulation is as follows, on the assumption that the hydrazide in alkaline solution is in the enol form and ionized although no titration curve is presented:



Reaction 1 leads to the release of nitrogen and need not be luminescent. Reaction 2 may be the major source of luminescence. This, unlike most previous ideas, suggests that the hydrazide is not the substrate but a catalyst for the decomposition of hydrogen peroxide, and it has been so considered in some kinetic studies.²⁵ A study of the reactions of the isolated hydrazide peroxide, including quantitative determinations of nitrogen and oxygen, should be revealing.

Stross and Branch,²⁴ also in 1938, on the basis of quantitative studies on the reactants, ferricyanide and hydrogen peroxide, and accounting for the oxygen, concluded that the chemiluminescence was associated with a two-step oxidation, probably to the azo compound postulated by Albrecht.⁸ In contradiction to Albrecht and in agreement with Drew,⁴¹ they consider that the luminescence comes before the breaking of the hydrazide ring. They consider that in their reaction system the ferricyanide produces a one-unit oxidation to a free radical which then reacts with hydrogen peroxide.

Additional proposals and experiments have been contributed by others.^{21, 25, 49} Weiss⁴⁹ combines a number of chemiluminescences into a single scheme. Lophine, after hydrolysis, luciferin, and the hydrazide have fairly labile hydrogen atoms which, he states, can be removed by oxidation. In general, Weiss says that the reaction can be written, $AH_2 \rightarrow AH \rightarrow A$, where A represents all of the compound except the two labile hydrogen atoms. This formulation also has the free radical, AH , as an intermediate step. The actual luminescence arises, according to Weiss's theory, by combination of radicals or ions:



Weber and his collaborators²¹ include both the peroxide of Drew⁴⁰ and Albrecht's⁸ system in a combined series of reactions.

It is evident that the problem has been left in a confused state, perhaps because of the war. Most authors, except Drew, include the azo compound to which Kautsky has suggested that he has a direct approach, but for which he has published only fragmentary evidence. Drew's isolation of the peroxide, on the other hand, seems definitive, but limited qualitative and quantitative data have been published upon its relation to the luminescent reaction, action of catalysts, etc. All formulations seem highly provisional until these basic matters and the fundamental position of the hydrazide in the luminescent reaction are settled.

The Biacridinium Salts

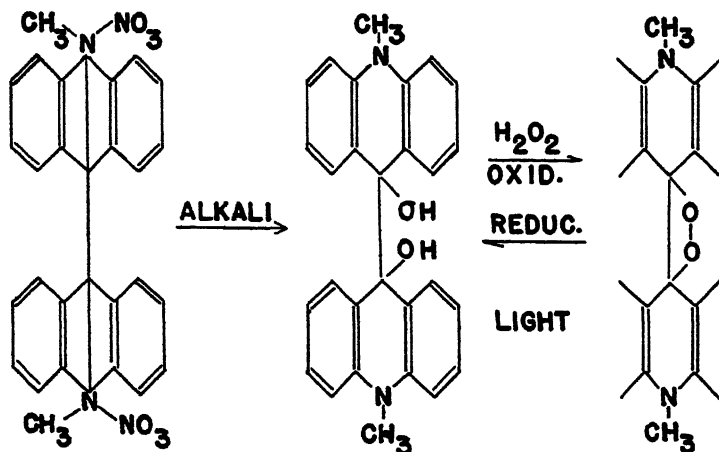
Gleu and Petsch,⁴ in 1935, showed that N,N'-dimethylbiacridinium nitrate* gives a chemiluminescence when treated with hydrogen peroxide in alkaline solution. The chemiluminescence is green, as is the fluores-

* Also called lucigenin.

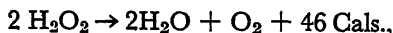
cence. The compound is yellow. The intensity of the light emitted varies widely, depending upon the conditions. In solutions made alkaline with sodium hydroxide, the luminescence is relatively dim when hydrogen peroxide is added, but it lasts for a long time. The intensity can be increased enormously by the addition of osmium tetroxide as a catalyst and the time of luminescence decreased to one second. Under such conditions, light could be observed at a concentration of luminescent substance of 10^{-10} M. When the reaction is carried out in a medium of concentrated ammonium hydroxide, the light with hydrogen peroxide alone is much brighter than in a sodium hydroxide solution. It may then last for only a minute. Osmium tetroxide has less effect on the reaction in concentrated ammonia. The quantitative effects of temperature,⁵¹ the kinetics of the reaction,^{25, 26} and the quenching or enhancing effects of foreign substances^{30, 52} have been studied.

Unexpectedly, when Gleu and Petsch tried other oxidizing agents, they observed no luminescence. However, they found that a number of common reducing agents, such as hydrosulfite or stannite, produced a chemiluminescence if oxygen was also present. None was observed if the solution was first freed of oxygen by passing nitrogen through it. In view of the need for oxygen in order to obtain luminescence, the authors concluded that the reaction was both an oxidation and a reduction. Since hydrogen peroxide is both an oxidizing and a reducing agent, this was not inconsistent with the other findings.

A tentative formulation designed to explain these several facts was given as follows:



According to this idea, the biacridinium salt would function essentially as a catalyst. With hydrogen peroxide, the net reaction would be:



and with the other reductants, the oxidation of the reducing agent by atmospheric oxygen. One difficulty immediately arises, since the 46 Cals. are about 10 Cals. too small for some of the wavelengths represented in the light emission. However, Gleu considers that this does not definitely prove the reaction to be inadequate.

Comparisons of the spectral distribution curves of fluorescence and chemiluminescence have also been made for the biacridinium salts. The compound fluoresces in water and in acid solution. According to Weber, it will also fluoresce in mildly alkaline sodium carbonate solutions but, like the hydrazide, the chemiluminescence and fluorescence appear to have widely different pH optima. The original dimethylbiacridinium compound was studied by Eymers and Van Schouwenberg,²⁸ who concluded that the fluorescent and chemiluminescent spectra were identical, even though the media in which they were studied were not identical. These same authors have extended their work by comparing fluorescent and chemiluminescent spectral distribution curves, including bioluminescent sources, after analysis. The analysis was based on the assumption that the observed graph, when intensity was plotted against frequency, was made up of two or more essentially symmetrical curves. The frequencies of these postulated fundamental curves tended to be the same for different reactions. How much the results depend on the above described secondary fluorescence is unknown.

More recently, however, Gleu and collaborators⁵³⁻⁵⁵ have prepared a number of biacridinium derivatives and compared the color of the fluorescence and chemiluminescence.⁵⁵ The diethyl derivative shows identical green fluorescence and chemiluminescence. The same is true for the diphenyl at some concentrations. However, below 10^{-6} M, the chemiluminescence becomes blue. Fluorescence and chemiluminescence also differ in other compounds. The authors conclude, therefore, that the supposed agreement, which they and others had previously reported and used as a partial basis for the above formulation, is merely a coincidence which occurs with some compounds.

Kautsky and Kaiser,⁵⁰ in 1943, reported that all previous interpretations of the spectral distribution curve of the chemiluminescence are faulty. As ordinarily obtained, they consider the light emission to be a mixture of a true chemiluminescence and the green fluorescence of the biacridinium nitrate. They state that when the reaction is carried out in a dilute solution at 40–50° C., the fluorescence is largely absent and the true chemiluminescence, blue in color, is obtained. This seems to fit the observations of Gleu and Schaarschmidt⁵⁵ mentioned above. Further, Kautsky and Kaiser find that the spectral distribution curve of this "true chemiluminescence" is closely similar to the fluorescence of N-methyl-acridone under exactly the same conditions. It is the acridone, therefore, which these authors consider as being the primary emitter of the

luminescence, and they regard its formation from the carbinol with liberation of 65 Cals. as the chemiluminescent reaction. If this is true, there should be a striking effect of pH on the color of the emission curve as usually obtained, since the fluorescence of the biacridinium salt is reported as decreasing in alkaline solution. Also, the question arises as to how the reducing agents produce their effect, since the chemiluminescent reaction, as formulated with hydrogen peroxide by these authors, is an oxidation.

Unfortunately, no additional work has been found, so that here, also, the fundamental role in the luminescent reaction, substrate or catalyst, of the major compound is in doubt. The formation of acridone as a possible side reaction was mentioned by Gleu and Petsch.⁴

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THE CHEMISTRY OF CYPRIDINA LUCIFERIN

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Luminescence is encountered throughout the animal kingdom and in certain lower forms of plant life. However, the luminescent reaction has been demonstrated *in vitro* in only five orders of animals. These include certain beetles (fireflies), one mollusc (*Pholas*), certain ostracods (e.g., *Cypridina*), a few worms (e.g., *Odontosyllis*), and at least one decapod, a deep-sea form. The most favorable organism as a source of material for the study of the luminescent reaction *in vitro* is the ostracod crustacean, *Cypridina hilgendorfii*, whose eminent suitability for the extraction of the compounds concerned was pointed out by Harvey (1917). This animal possesses a gland in the head region, where luciferase, the enzyme which catalyzes the reaction, and luciferin, the substrate, are produced. When alive, the animal can eject these two compounds, in the form of two kinds of granules, into the sea water through separate pores, and a blue luminescence then occurs in the water. If the organisms are dried immediately after being caught, and are kept dry, the luciferin and luciferase in the gland remain stable for years and can be extracted when desired. The paper by Harvey (1948) contains photographs of *Cypridina* and drawings of the gland, on page 335.

The simplest method of extracting luciferin from *Cypridina* is by grinding up the dry organisms and adding hot water. This inactivates the luciferase, so that no luminescence occurs in the extract but the luciferin is obtained unaltered. The suspension is immediately cooled to retard destruction of the luciferin by oxidation. After filtering, the clear solution contains luciferin and all the other water-soluble components of the organism. Such crude luciferin extracts, though easy to prepare, are not very suitable for chemical work, because of the great instability of the luciferin in the presence of dissolved oxygen and of oxidizing systems that are extracted along with the luciferin. Also, the impurity of such preparations is undesirable from a chemical point of view and, indeed, luciferin in crude extracts often behaves quite differently than does more highly purified luciferin.

Several methods of extracting and purifying *Cypridina* luciferin have been developed. Kanda (1924, 1929) extracted the dry, powdered organisms with methyl alcohol in absence of oxygen and, after various precipitations and re-solutions, obtained luciferin which was more stable against oxidation and certainly purer than that in a crude aqueous extract. The chemical work on luciferin prior to about 1940 is discussed in publications of Harvey (1940, 1941).

The greatest advance toward the purification of luciferin is due to the work of Anderson. To determine quantitatively the concentration of luciferin present in his various purification steps, he developed a photoelectric method for measuring the total light emitted by a luciferin solution. This apparatus (Anderson, 1933) does not measure light intensity directly, as do most photoelectric and all visual methods, but instead it yields the integral curve for the luminescent reaction. The output of the photoelectric cell is stored in a condenser and the charge accumulated on the condenser, after any time from the start of the reaction, can be balanced with a potentiometer, using a Lindemann electrometer. The method is therefore a null-point one, and light-emission can be measured in terms of millivolts, to about one millivolt, the limit set by the Lindemann electrometer. The capacity of the condenser determines the sensitivity of the method. With this apparatus, very weak luminescences can be measured quantitatively. A good example of the sort of data obtainable is given in FIGURE 2. The value of the ordinate at any time is a relative measure of the amount of luciferin that has reacted with luciferase to give luminescence, from the start of the reaction to that time. The slope of the curve at any time is proportional to the intensity of the luminescence at that particular time. The data secured by the method are, therefore, analogous to those obtained in any reaction where an end product is determined quantitatively at various times during the course of the reaction.

Anderson (1935) worked out a purification procedure which yields from *Cypridina* a luciferin of much greater purity than had been obtained previously. Briefly, the method is as follows. Dry, powdered organisms are extracted for twenty-four hours with absolute methyl alcohol that is kept free of dissolved oxygen by saturation with purified hydrogen in a special extraction vessel. The vessel is then opened and a small amount of *n*-butyl alcohol is added and the methyl alcohol is removed by evaporation at low pressure. This solution, after having been chilled, is treated with benzoyl chloride. The resulting derivative of luciferin not only does not give light on addition of luciferase, but is much more stable in the presence of air than is the luciferin in its original state. Water is now added to hydrolyze the excess benzoyl chloride, and the butyl alcohol fraction is dissolved in 10 volumes of water. The resulting solution is extracted with ether, into which most of the butyl alcohol and luciferin derivative pass, leaving highly colored impurities in the aqueous phase. The ether is next removed *in vacuo*, leaving the inactive luciferin, already considerably free of colored impurities, in the residual butyl alcohol. This solution is mixed with a large volume of 0.5 *N* HCl, saturated with hydrogen, and heated in a hydrogen atmosphere for an hour at 95°-100° C and then cooled in an ice water bath. The mixture, now containing

active luciferin, is then washed again with ether. At this stage, the "reactivated" luciferin remains almost entirely in the aqueous phase, and considerable colored material passes into the ether phase. The luciferin is finally extracted from the aqueous solution with *n*-butyl alcohol, deaerated with hydrogen and, if desired, is put through the same cycle of purification a second time. Some yellow color remains even after three cycles of purification and, as will be apparent presently, this is undoubtedly a property of luciferin.

This method results in a purification of the luciferin of about 2,000 times, in terms of amount of light per unit of dry weight of solid material in the final solution as compared with the starting material. The stability of the luciferin against oxidation is also greatly increased, perhaps because oxidizing systems that were present in the original material have been removed during the purification procedure.

In most of the recent work which has been done on the chemistry of luciferin, the luciferin has first been subjected to Anderson's (1935) purification procedure. It has frequently been found that quite different experimental results are obtained when using this purified material than with crude aqueous extracts of *Cypridina*. For example, luciferin in crude extracts is not affected by even high concentrations of cyanide, whereas the more purified compound loses its light-producing characteristics in the presence of extremely low cyanide concentrations. (Giese and Chase, 1940.)

The luciferase used with this purified luciferin, when studying the luminescent reaction *in vitro*, is ordinarily prepared by dialyzing a water extract of powdered dry *Cypridina* against distilled water for some days at a low temperature until the solution has become practically colorless. Considerable inactive protein is precipitated by this treatment and most of the luciferin and other dialyzable compounds are certainly eliminated. That luciferin can pass through a dialysis membrane was demonstrated by Harvey (1917), and it seems reasonable to assume that oxidized luciferin is also dialyzable, although this has not actually been shown.

Anderson found (1936), with luciferin purified by his method (1935), that the luminescent reaction of luciferin and luciferase, which requires the presence of oxygen, was irreversible. On the other hand, he found that the non-luminescent oxidation of luciferin which occurs in the presence of dissolved oxygen or of certain oxidants (*e.g.*, ferricyanide) can be almost completely reversed by $\text{Na}_2\text{S}_2\text{O}_4$ or suitable reductants, if the latter are added soon enough. Anderson concluded that the luminescent reaction of luciferin and luciferase and the non-luminescent oxidation of luciferin are quite different from each other. He found a redox potential for the reversible oxidation of luciferin, assuming that the oxidation removed two electrons per molecule, about 0.01 volt negative to quinhydrone at 23° and pH 6.8 (Anderson, 1936). Korr (1936) found a

similar value. E_0' is, then, about +0.260 volt at pH 7. Anderson pointed out the similarity of this value to the redox potentials encountered in the case of certain naturally occurring polyhydroxybenzene derivatives studied by Ball and Chen (1933). He has emphasized the possibility that such a grouping may be present as part of the luciferin molecule. It is interesting to note that, in the work of Ball and Chen, the oxidized forms of the natural compounds whose potentials they measured were very unstable. In fact, it was necessary to use a flow technique in order to make the measurements. This feature of these compounds again shows a similarity to the luciferin-oxidized luciferin system, where the oxidized luciferin also seems to be unstable, since it cannot be reduced if it has stood too long in the presence of an oxidizing agent or of dissolved oxygen.

Anderson (1936) studied the reversible non-luminescent oxidation of purified luciferin in some detail and found that the luminescent reaction which occurred upon adding luciferase to a solution of luciferin that had been exposed to air for a short time showed evidence of two distinct kinds of luminescence, a rapid and a slow luminescence, occurring simultaneously. He attributed the intense, rapid luminescence to reduced luciferin in the solution and the dim, slow luminescence to reversibly oxidized luciferin which was being reduced in the presence of luciferase or of compounds extracted with it.

The oxidation of luciferin can be accelerated by irradiation with certain parts of the spectrum, as was shown by Harvey (1925, 1926). He used crude luciferin extracts from *Cypridina* and found that blue, violet, and near ultraviolet light were all effective in accelerating the oxidation. Chase and Giese (1940) later found that, when purified luciferin was used, the ultraviolet below 3000 Å was effective, but ultraviolet of longer wavelength and the visible part of the spectrum were not, unless a photo-sensitizing compound was added. A number of compounds were found to act as sensitizers, including eosin and fluorescein. Boiled, crude aqueous extracts of *Cypridina*, containing oxidized luciferin and all the other water-soluble components of the organism, were very effective, indicating that impurities in the crude extract were sensitizing the oxidation of luciferin irradiated by visible light in the case of Harvey's earlier experiments.

Chase and Giese found that, when the luminescent reaction was measured upon adding luciferase to luciferin that had been irradiated with ultraviolet light or with visible light in the presence of a sensitizer, the reaction could be differentiated into two parts, one a bright luminescence and the other a dim luminescence, as Anderson had found in the case of luciferin that had stood in the presence of dissolved oxygen. An interesting exception occurred when the purified luciferin was irradiated with

visible light in the presence of riboflavin as a sensitizer. In this case, the luminescent reaction which ensued on addition of luciferase lacked the dim component, as shown in FIGURE 1. Riboflavin evidently prevents the

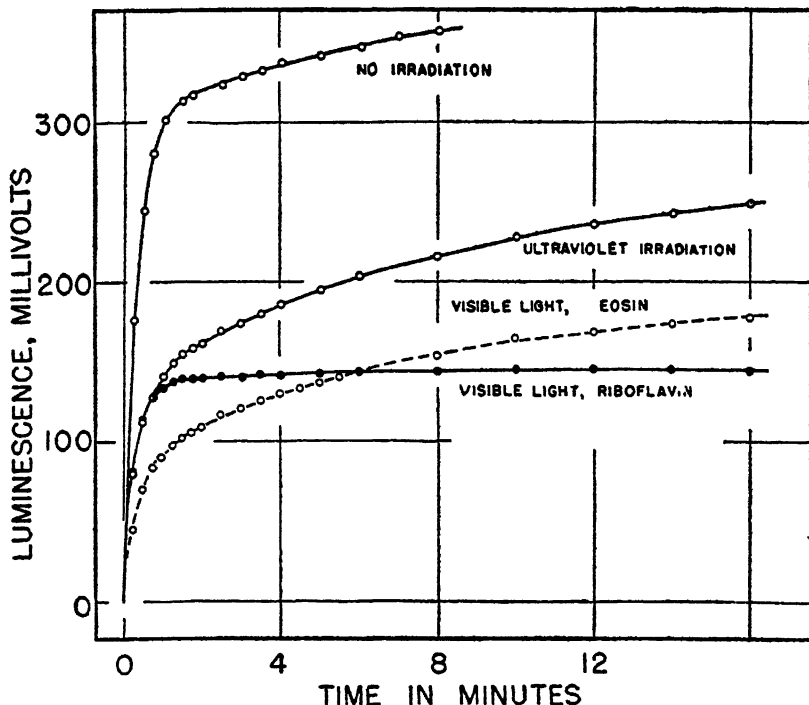


FIGURE 1. Typical luminescence curve obtained on adding luciferase to luciferin solutions which have been irradiated for three minutes with visible light in the presence of about 0.001 per cent concentration of riboflavin. Although an initial bright luminescence occurs, there is no light-emission after two minutes. The luminescence curves obtained after irradiation of luciferin with visible light with eosin present, and after ultraviolet irradiation without a sensitizer, both show not only a bright luminescence during the first two minutes, but also a dim luminescence which persists for at least sixteen minutes.

reduction by luciferase (or whatever is responsible) of reversibly-oxidized luciferin. However, riboflavin is effective in this way only if the oxidation of luciferin is caused by the action of light with the flavin as sensitizer. If the luciferin is oxidized by dissolved oxygen, in the dark, with riboflavin present in the solution, and luciferase is then added, the resulting luminescence shows the bright and dim component ordinarily observed.

Recently, Chase and Lorenz (1945) separated the velocity constants of the enzyme-catalyzed luminescent reaction of luciferin and of its non-luminescent oxidation. They measured the luminescent reaction at five temperatures and fitted the data with an equation representing two first-order reactions occurring simultaneously. FIGURE 2 shows their experi-

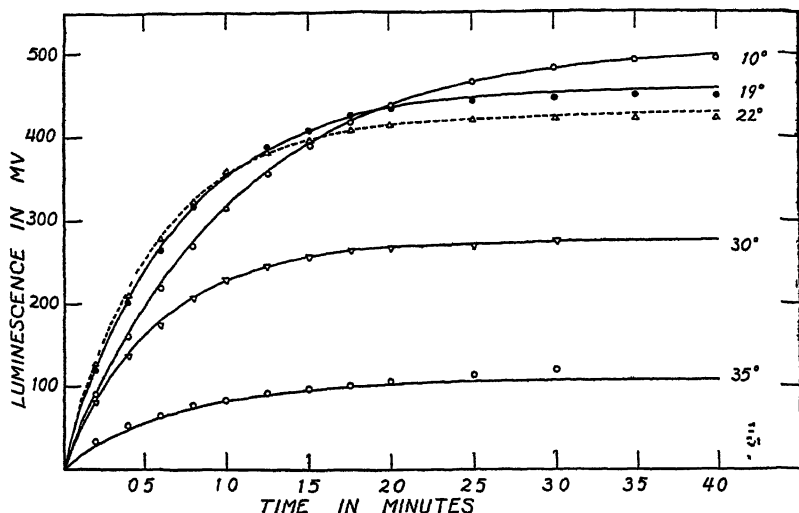


FIGURE 2. The luminescent reaction at five temperatures. The symbols represent experimentally measured total light emitted from the moment of mixing luciferin and luciferase to various times. The curves are theoretical, calculated from an equation derived on the assumption that two first order reactions are occurring simultaneously one, the luminescent reaction of luciferin, and the other, its non-luminescent oxidation.

mental points and the curves which describe them are theoretical. Since Anderson (1936) has demonstrated that the luminescent reaction of luciferin and luciferase is a different process from the non-luminescent oxidation of luciferin, it is clear that both these processes may occur simultaneously. Chase and Lorenz assumed that, since dissolved oxygen is probably present in excess, the non-luminescent oxidation of luciferin might obey a first-order equation even though it might actually be a bimolecular reaction. Amberson had shown (1922) that the luminescent reaction obtained on mixing crude *Cypridina* luciferin and luciferase extracts obeyed the equation for a first order reaction. A record of the logarithmic decay of *Cypridina* luminescence is shown in the paper by Harvey (1948). The equation which describes the curves of FIGURE 1 was derived by Chase and Lorenz on the assumption, then, that the luciferin is the principal substrate in two first-order reactions occurring simultaneously, only one of which results in light emission. If x is the luciferin consumed by the luminescent reaction after time, t , and y is the luciferin consumed by the non-luminescent oxidation reaction after time, t , and if k_1 and k_2 are the respective velocity constants and a is the concentration of luciferin initially present, then:

$$\left\{ \begin{array}{l} \frac{dx}{dt} = k_1 (a - x - y) \\ \frac{dy}{dt} = k_2 (a - x - y) \end{array} \right\} \text{are to hold simultaneously.}$$

This gives $\frac{dy}{k_2} = \frac{dx}{k_1}$, whence, since $y = 0$ when $x = 0$, $y = \frac{k_2}{k_1} x$.

Using this value of y , $\frac{dx}{dt} = k_1 \left(a - x - \frac{k_2}{k_1} x \right)$.

On integration and simplification, one obtains

$$x = \frac{k_1 a}{k_1 + k_2} \left(1 - e^{-(k_1 + k_2) t} \right)$$

This equation describes the data of FIGURE 2 rather exactly, the values of k_1 and k_2 being obtained directly from the experimental measurements.

If the logarithm of k_2 (the velocity constant of the non-luminescent oxidation reaction) be plotted against the reciprocal of the absolute temperature, a fairly straight line is obtained, as shown in FIGURE 3, and

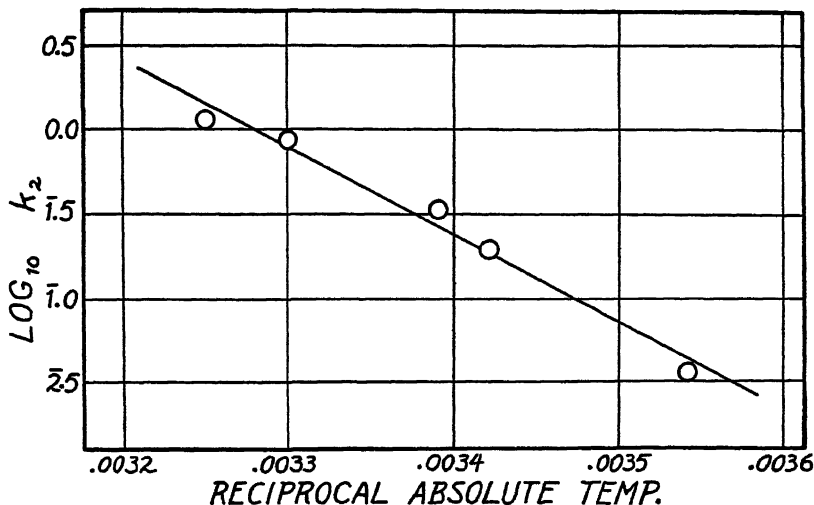


FIGURE 3. Logarithm₁₀ of the calculated velocity constant of the non-luminescent oxidation of luciferin plotted against the reciprocal of the absolute temperature. The slope represents a value of about 25,000 calories for the energy of activation.

the slope of this line corresponds to an energy of activation of about 25,000 calories per mole. The velocity constant of the luminescent reaction, on the other hand, has a maximum value at about 23° C, the principal effect of temperature in this case being upon the enzyme, luciferase.

Although the subject of this paper is the chemistry of luciferin, rather than the kinetics of the luminescent reaction, it seems proper to include one more datum from the kinetic point of view, in addition to those already mentioned.

Chance, Harvey, Johnson, and Millikan (1940) measured, by means of a special technique, the luminescent reaction of unpurified *Cypridina* luciferin and luciferase mixed in two different ways. In the first method, luciferin, in solution containing dissolved oxygen, was mixed with luciferase, also in solution containing oxygen. In the second method, a de-aerated solution containing both luciferin and luciferase was mixed with a solution containing dissolved oxygen. The luminescent reaction was much faster when the luciferase and luciferin were previously mixed than it was in the other case. The data were interpreted as showing that a combination between luciferin and luciferase is an essential requirement for luminescence, and that this combination represents a relatively slow reaction. The analysis also showed that the enzyme, luciferase, is the light-emitting molecule. This latter interpretation has generally been made, although it has not been proved that the light-emitting molecule may not be luciferin.

So far as the chemical structure of *Cypridina* luciferin is concerned, very little is known with certainty. However, several hypotheses have been advanced, most of them backed by some experimental evidence. The method of purification rules out any possibility that luciferin is a protein, while the redox potential measurements mentioned above have shown a possible relationship to hydroxybenzene derivatives. Anderson (1936) also interpreted the formation of benzoyl derivatives of luciferin as indicating the presence of reactive hydrogen which might be attached to nitrogen, sulfur or oxygen. Chakravorty and Ballentine (1941), however, found no nitrogen or sulfur in luciferin.

Giese and Chase (1940) found that luciferin purified by Anderson's method, irreversibly lost its property of giving luminescence with luciferase when very small concentrations of cyanide were present, as illustrated in FIGURE 4. On the other hand, luciferin in crude extracts of *Cypridina* is not affected by high cyanide concentrations. This indicated a chemical reaction between luciferin and cyanide, and Giese and Chase calculated a molecular weight for luciferin of between 800 and 2400, assuming the luciferin to be 100 per cent pure and assuming a 1:1 combination between the luciferin and the cyanide. Since the luciferin is certainly not pure, the true value for the combining weight is probably at least as low as 800 or even less. Giese and Chase interpreted the reaction with cyanide as due to cyanhydrin formation, and assumed an aldehyde or keto group on the molecule, which group might also be the site of combination with luciferase in the irreversible luminescent reaction.

With a view toward obtaining corroborative data on the combining weight of luciferin, an attempt was made recently, in collaboration with Dr. C. B. Anfinsen (unpublished), to determine an oxygen uptake by purified luciferin during its non-luminescent and luminescent oxidations, using the Cartesian diver method, and assuming two atoms of oxygen

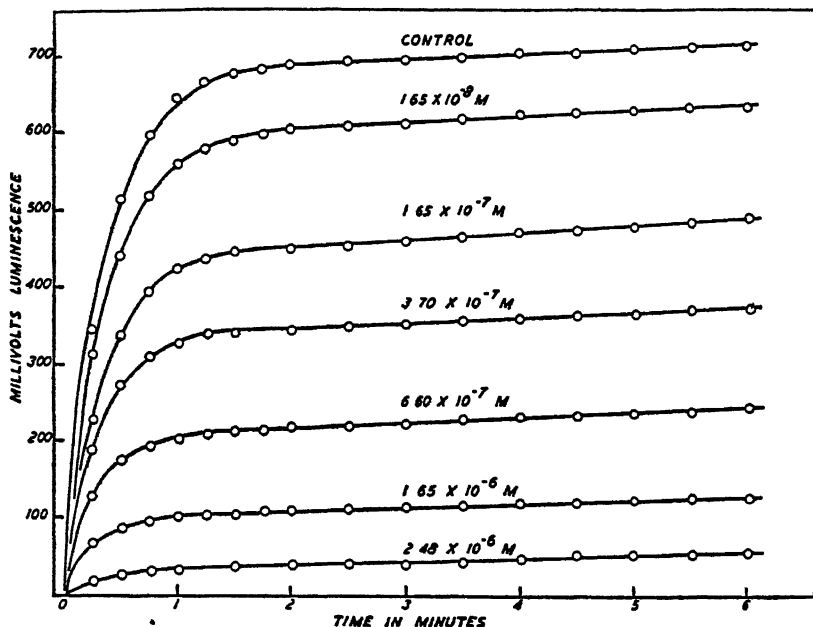


FIGURE 4 Quantity and rate of light-emission from identical samples of luciferin which were treated with various concentrations of cyanide, indicated on the curves, before addition of luciferase. Since the velocity constants of the curves are essentially the same, the luciferin rather than the luciferase is affected by the cyanide.

combined per molecule of luciferin in the non-luminescent oxidation. Although there were indications of oxygen uptake in some cases, the results were inconclusive. Parallel tests on the stability against oxidation of comparably small samples of luciferin solution, made it appear likely that the combination with oxygen, assuming that it does occur, took place during the time the divers were being filled and before the actual measurements of gas uptake could be started. R. S. Anderson has unpublished data on the concentration of ferricyanide necessary to oxidize known amounts of luciferin, which indicate a combining weight of about the same order of magnitude found in the case of the cyanide experiments (personal communication). The uncertainty in all measurements of the combining weight of luciferin by such methods lies chiefly in the assumption as to the purity of the luciferin. The cyanide data of Giese and Chase certainly point to a combining weight (and probably molecular weight) of less than 1000, perhaps about half this value. There seems little doubt that luciferin is a relatively small molecule.

Chase found (1942) that the luminescent reaction of purified luciferin and luciferase was reversibly inhibited by sodium azide. Since the total light was affected but not the velocity constant, the action of the azide was attributed to a reversible combination with the luciferin rather than

with the luciferase. As was pointed out in another connection by Johnson, Eyring, and Williams (1942; see page 259), this interpretation may not be justified in the case of a reversible combination. The reaction might equally well take place between the azide and luciferase, although this does not seem likely. FIGURE 5A shows the effect of azide concentration upon the total light emitted. Experiments were run at pH 5.4 and at pH 6.6 and, as the figure shows, the azide was more effective at the lower pH. Since the pK of hydrazoic acid is about 4.7, a higher concentration of the undissociated acid would be present at pH 5.4 than at pH 6.6. For this reason, the effect was attributed to the undissociated acid rather than the azide anion.

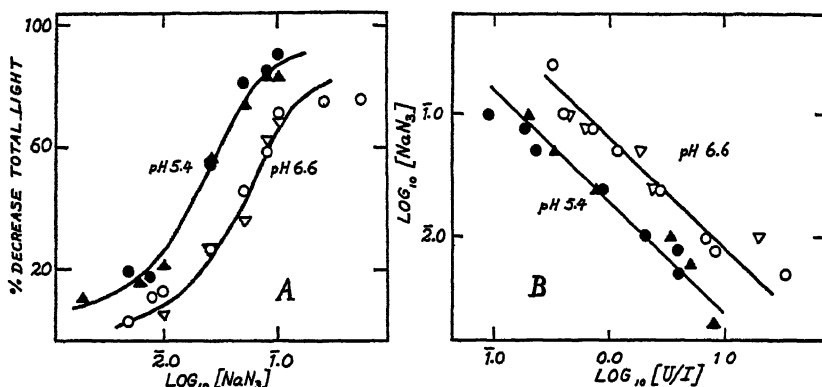
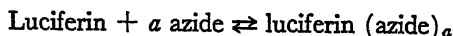


FIGURE 5. A. Per cent decrease of total light plotted against the logarithm of the sodium azide concentration. Four series of experiments are shown, two at pH 6.6 and two at pH 5.4. A given azide concentration is more effective at the lower pH, indicating that HN_3 may be involved.

B. The same data plotted in terms of a mass law equation. The slopes of the lines are approximately unity, which may indicate that one azide molecule or ion combines with a single luciferin molecule.

Since this reaction with azide is reversible, the data can be put into a form that makes them analyzable in terms of a mass law equation, as has been done in cases of inhibitor action on enzyme systems (see, for example, Fisher and Öhnell, 1940). In the present case, the analysis is as follows:



$$\frac{[\text{Luciferin}] [\text{Azide}]^a}{[\text{Luciferin (Azide)}_a]} = K$$

$$\frac{[\text{Luciferin}]}{[\text{Luciferin (Azide)}_a]} = K \cdot [\text{Azide}]^{-a}$$

If $[\text{Luciferin}]$ is called U and is put equal to the percentage of luminescence that occurs at any given azide concentration, taking as 100 per cent

the luminescence obtained when no azide is present, and if [*Luciferin* (*Azide*)_a] is called *I* and is put equal to the percentage of potential luminescence that does not occur, then,

$$\frac{U}{I} = K \cdot [\text{Azide}]^{-a}, \text{ or } \log \frac{U}{I} = -a \log [\text{Azide}] + \log K.$$

Therefore, by plotting $\log_{10} \frac{U}{I}$ against the \log_{10} of the azide concentra-

tion, a straight line should be obtained and the slope of the line should give the value of *a*, the number of azide molecules which combine with a single luciferin molecule. FIGURE 5B shows the data plotted this way. It is apparent that the slope is about -1, indicating one molecule of hydrazoic acid combining reversibly with each molecule of luciferin.

Fieser and Hartwell (1935) studied the reaction of hydrazoic acid with benzo- and naphthoquinones. According to them, an azido-hydroquinone is first formed, changing to an amino-quinone with liberation of nitrogen. Although a reaction of this sort would hardly be considered as readily reversible, it seems possible that a related type of reaction might occur in the case of luciferin and, if so, would be further evidence for a quinonoid structure.

Chase has shown (1945) that luciferin, freshly dissolved in pH 6.8 phosphate buffer in absence of air, has an absorption band at about 435 mμ in the visible spectrum. Measurements of the absorption spectrum were made with a recording spectrophotometer (Hardy, 1935). If dissolved oxygen is present, this band is rapidly replaced by another at about 465 mμ and this latter band then disappears slowly, leaving a practically colorless solution (Chase, 1943). These changes in the visible absorption spectrum of luciferin solutions take place much more slowly if the pH is more acid than 6.8. For example, as FIGURE 6 illustrates, at pH 5.1 the change is only about one-fifth as fast. Furthermore, at pH 5.1 there is definite evidence, from the absorption spectrum, for the production of a compound having acid-base indicator properties during the exposure of the luciferin to dissolved oxygen. This compound absorbs more strongly in the violet and near ultraviolet at a pH of 5.1 than it does at pH 6.8, as is also shown in the curves of FIGURE 6. The rate of change of the absorption spectrum increases with increase of pH in about the same way that the rate of non-luminescent oxidation of luciferin varies with pH in solutions containing dissolved oxygen (Chase, 1940). In fact, there is little doubt (Chase, 1943) that the amount of labile color in a luciferin solution is directly proportional to the concentration of luciferin, as measured by the total light obtainable from the solution.

If the visible absorption spectrum of a luciferin solution is measured during the luminescent reaction of luciferin and luciferase (as can be done with the Hardy recording spectrophotometer, which is not affected

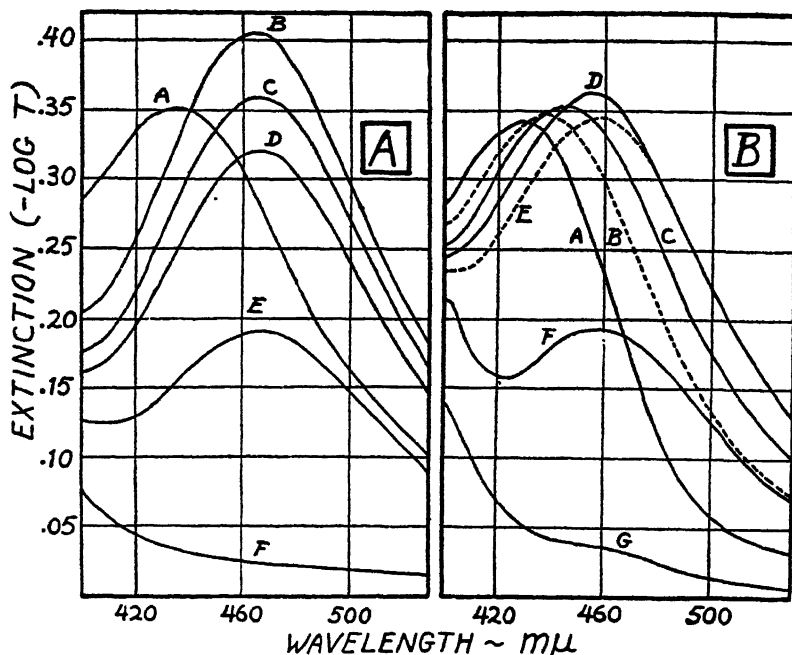


FIGURE 6. The left half shows the changes which occur in the visible absorption spectrum of a luciferin solution at pH 6.8 during exposure to air. Curve A is the spectrum 5 minutes after the luciferin was dissolved. Curve B was measured after a total elapsed time of 19 minutes, curve C after 81 minutes, curve D after 44 minutes, curve E after 81 minutes, and curve F after 2 days.

The right part of the figure shows similar measurements on a luciferin solution at pH 5.1. Curve A was measured 4 minutes after the luciferin was dissolved, curve B after 18 minutes, C after 30 minutes, D after 59 minutes, E after 81 minutes, F after 300 minutes, and G after 26 hours.

by continuous light-emission in the sample), it is found that the same changes take place, but that they occur one hundred times as fast as during the spontaneous, non-luminescent oxidation of luciferin in absence of the enzyme. Absorption spectra measured during the luminescent reaction and during the non-luminescent oxidation of luciferin are compared in FIGURE 7.

It seems possible that the shift of absorption maximum from 435 $m\mu$ to 465 $m\mu$ may be correlated with the reversible oxidation of luciferin demonstrated by Anderson (1936). The two facts that, first, the products of the luminescent reaction of luciferin and of its reversible, non-luminescent oxidation are evidently different (Anderson, 1936) and that, second, the changes in the absorption spectrum are the same during the two reactions (Chase, 1943), would not appear to support such an interpretation. However, the two reactions might be different without necessarily causing a difference in the light-absorbing groups of the molecule. Further experimental evidence is needed before more than tentative conclusions

can be drawn. If the assumption be granted that the shift in spectral absorption actually represents the reversible oxidation, then this might indicate the oxidation of a polyhydroxybenzene derivative or related compound. So far, no attempt has been made to demonstrate the reverse shift of the absorption band from $465\text{ m}\mu$ to $435\text{ m}\mu$ as a result of reduction of spontaneously oxidized luciferin.

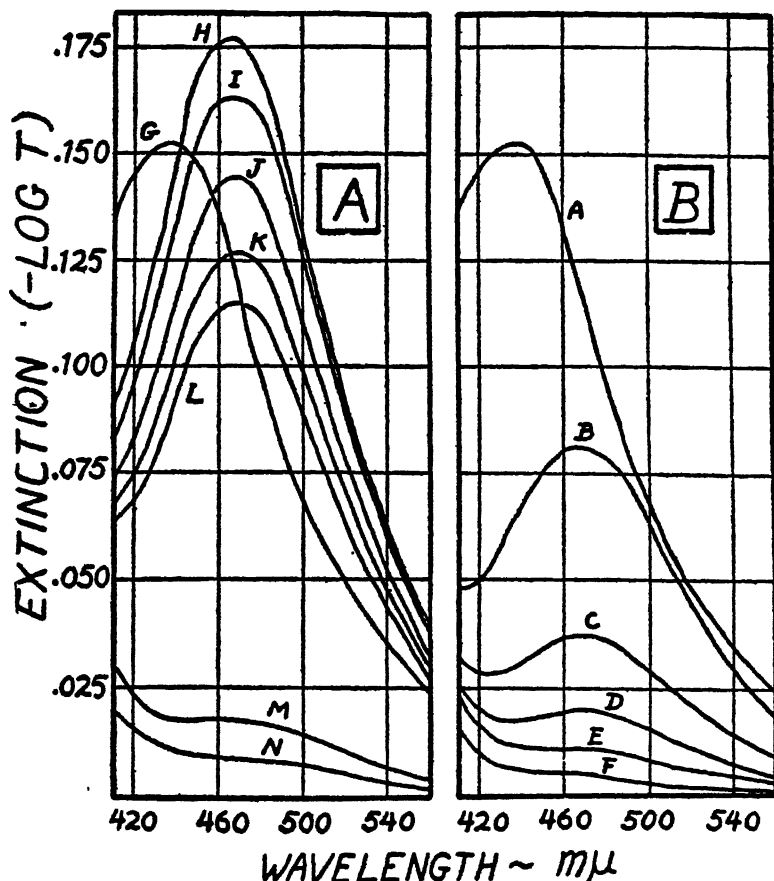


FIGURE 7. The curves shown in the left half of the figure are absorption spectra of a luciferin solution measured during exposure to air. Curve G is the spectrum immediately after the luciferin was dissolved; H, I, J, K, L, M and N, after 9, 20, 30, 39, 49, 465 and 1140 minutes, respectively.

In the right half of the figure are shown absorption spectra of a luciferin solution in which luminescence is occurring due to the action of luciferase, the enzyme. Curve A is the spectrum immediately after the luciferin was dissolved, but before the luciferase was added. Curve B was measured 1 minute after the luciferase was added, curve C after 12 minutes, D after 22 minutes, E after 40 minutes and F after 457 minutes. The changes in the absorption spectrum are about 100 times as rapid during luminescent oxidation by luciferase as during non-luminescent oxidation.

The slow disappearance of the 465 $m\mu$ band upon prolonged exposure to dissolved oxygen, or its rapid disappearance in the presence of luciferase, may indicate the disruption of a ring structure. If this be true, it is not surprising that the luminescent oxidation of luciferin should be irreversible. As mentioned earlier, the oxidation products of the naturally occurring polyhydroxybenzene derivatives studied by Ball and Chen (1933) were also unstable compounds. It has been shown by Hooker (1936) that during oxidation by alkaline potassium permanganate, certain 2-hydroxy-1,4-naphthoquinone derivatives undergo an opening of the quinone ring followed by subsequent closure. If a similar reaction occurred in the irreversible oxidation of luciferin (although there is, of course, no experimental evidence for such a mechanism), it seems quite conceivable that the ring, having opened, might become so constituted that it could not close. This would be a possible explanation for the irreversible step in the oxidation, the reversible step having been the oxidation to a quinone.

Chakravorty and Ballentine (1941), in a short note, summarized the then available chemical data on *Cypridina* luciferin and suggested a partial structure for the molecule, illustrated in FIGURE 8. The authors

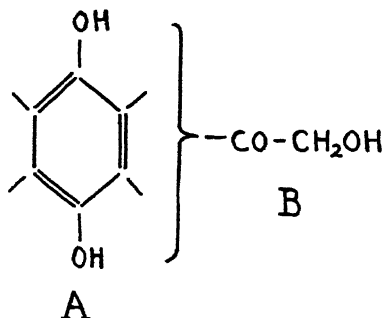
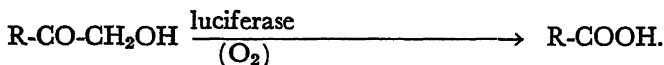
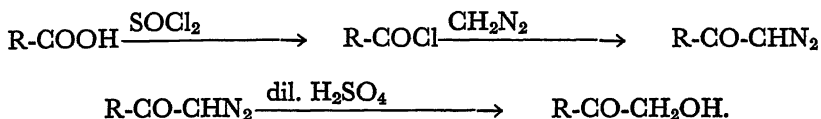


FIGURE 8. The partial structure suggested for luciferin by Chakravorty and Ballentine (1941). The group labeled "A" would represent the part of the molecule capable of reversible oxidation and reduction but not necessarily concerned in the luminescent reaction. The side chain labelled "B" would be capable of reacting with benzoyl chloride or cyanide or, perhaps, with luciferase, and could be oxidized to a carboxyl group as a result of the luminescent reaction with the enzyme.

found further evidence for a keto group from the fact that a microcrystalline precipitate was obtained upon treatment of luciferin with hydroxylamine acetate. They suggested that the irreversible luminescent reaction involved the oxidation of the side chain to a carboxyl group, as follows:



If this were correct, they reasoned that it should be possible to regenerate the starting material from the product of the luminescent reaction by appropriate chemical treatment, thus:



After having treated luciferase-oxidized luciferin in this way, they obtained a compound which, they believed, gave luminescence with luciferase, greater in intensity than was obtained from their control. Unfortunately, no quantitative measurement of the luminescence was made, and the light which was obtained was probably only a very small percentage of that which should have been expected, considering the high concentration of luciferase-oxidized luciferin in their starting material. The experiment is especially significant, however, in that it represents probably the first attack upon the problem of the structure of luciferin by the methods of organic chemistry. A continuation of this kind of approach should prove fruitful. Chakravorty and Ballentine also reported microchemical analyses of samples of purified luciferin, which showed no nitrogen, sulfur, halogen, or ash.

McElroy and Ballentine (1944) found evidence for the presence of acid-labile phosphate in purified luciferin. They also reported a significant increase in inorganic phosphate during the luminescent reaction, and stressed the possibility that formation of an energy-rich phosphate bond may be involved in the luminescent reaction. Eymers and van Schouwenburg (1937) had measured the emission spectrum of crude extracts of *Cypridina* luciferin and luciferase. Their analysis showed two fundamental frequencies: one of 21,250, corresponding to a wavelength of 470 $m\mu$, and another smaller component of 18,200, corresponding to a wavelength of 549 $m\mu$. These frequencies represent energies of about 59,000 and 50,000 calories, respectively, and, according to McElroy's and Ballentine's calculations, the preservation of energy as phosphate bond energy from oxidation of the postulated side chain of luciferin, and the subsequent release of this energy through reaction with luciferase would be sufficient to produce luminescence having the observed spectral quality.

If luciferin does have an hydroxybenzene, or related, structure and if it can be reversibly oxidized to a quinone, measurements of its ultraviolet absorption spectrum would be expected to throw light on the specific configuration of the molecule. The only such measurements so far recorded (Chase, 1943; Chase and Giese, 1940) cannot be regarded too seriously, since impurities were certainly present in the luciferin solutions even after two cycles of purification by Anderson's procedure (1935). Impurities would doubtless cause much greater interference with the

ultraviolet spectrum than in the visible. An ultraviolet and visible absorption spectrum of luciferin in pH 6.8 phosphate buffer is shown in FIGURE 9, measured immediately after dissolving the luciferin and after exposure

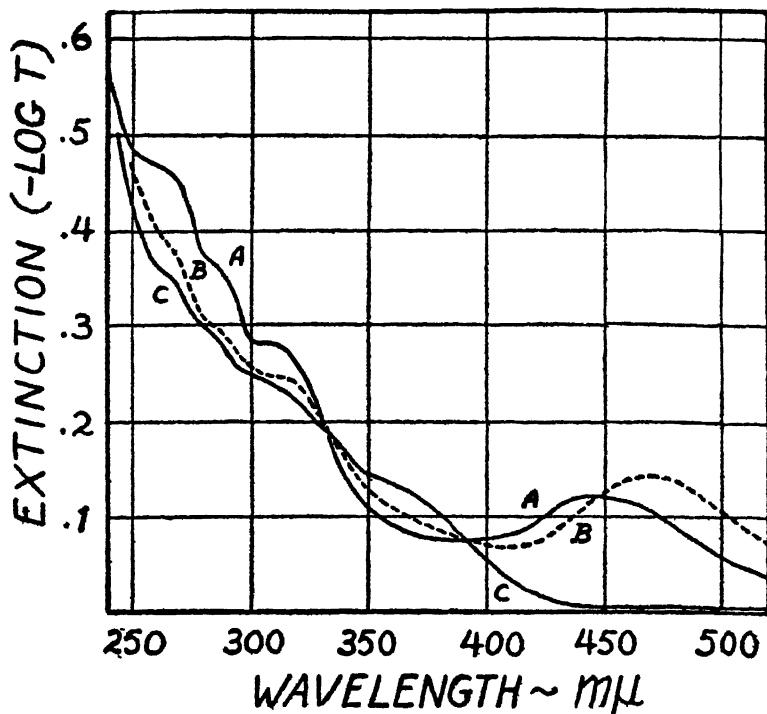


FIGURE 9. Visible and ultraviolet absorption spectra of a luciferin solution at pH 6.8. The spectra were recorded with the Harrison and Bentley spectrophotometer (1940). Curve A was measured as soon as possible after the luciferin had been dissolved. Curve B was measured after exposure of the solution to air for 32 minutes, and curve C after 24 hours.

to air during two time-intervals. A qualitative resemblance to the spectra of *p*-quinone and of certain naphtho- and anthraquinone derivatives actually seems to exist, and it is possible that luciferin may have an aromatic ring structure as its nucleus. Since the solvent frequently exerts a considerable effect on the absorption spectrum of a solution, precise measurements of the absorption spectrum of luciferin should be made, using a variety of solvents, in order to secure data susceptible of interpretation in terms of structure.

With the ultraviolet absorption spectrum measurements, such as they are, favoring *p*-quinone, naphthoquinone or anthraquinone derivatives, the first-mentioned structure seems most acceptable in view of the fact that the E_0' (pH 7) of the luciferin system is almost identical with that of quinhydrone (Anderson, 1936; Korr, 1936) and not sufficiently nega-

tive to fall in the range characteristic of naphtho- and anthraquinones. It must be remembered, however, that substitution in a quinone ring can greatly change the oxidation-reduction potential in either a positive or negative direction, depending upon the nature of the substituent group.

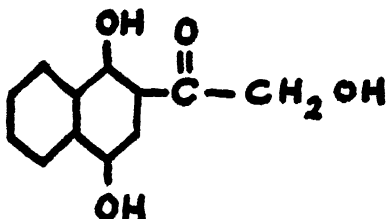
As stated earlier, luciferin and luciferase have not yet been extracted from luminous bacteria although there is every reason to believe that they occur. Recently Kluyver, van der Kerk, and van der Burg (1942) determined the action spectrum for the inhibition of bacterial luminescence by light of various wavelengths in the visible and ultraviolet. Assuming that this action spectrum represented the absorption spectrum of a compound closely related to bacterial luciferin, probably oxidized luciferin, they tentatively identified this compound from its spectrum, and from other considerations, as a 1,4-naphthoquinone derivative with a ketohydroxy group directly substituted in the quinone ring. They based their interpretation largely upon the conclusions of Chakravorty and Ballentine (1941), discussed above. There is a possibility, as the authors point out, that the action spectrum in this case may be affected by inert colored compounds in the medium or in the bacterial cell, or that it may represent the absorption spectrum of some compound which is so acting as to sensitize to light a component of the luminescent reaction. The action spectrum as measured might not, therefore, necessarily represent the absorption spectrum of oxidized luciferin. Although the actual comparison of the measured action spectrum with the absorption spectrum of the naphthoquinone derivative is not shown in their paper, it does appear in the doctorate thesis of van der Kerk (1942). While there is a qualitative resemblance between the two spectra as far as shape is concerned, the actual positions of absorption bands differ rather widely. Furthermore, the spectrum of the naphthoquinone derivative was measured in hexane, whereas the solvent for the hypothetical oxidized bacterial luciferin is unknown but is presumably largely aqueous. For these reasons, the conclusions as to structure should, as the authors themselves stress, be considered as purely tentative.

Another investigation on the luminescent reaction in bacteria which has yielded information as to certain characteristics of bacterial luciferin is that of Johnson, van Schouwenburg, and van der Burg (1939). They recorded the flash of luminescence in bacteria, following anaerobiosis under a variety of conditions. They concluded* that, since their data indicated that the luminescent oxidation of luciferin by luciferase and oxygen is evidently irreversible, whereas the dark oxidation by a substrate (or its breakdown product) is reversible, the situation is rather closely comparable to that in *Cypridina* extracts. Anderson (1936) had shown that the luminescent and non-luminescent oxidations of *Cypridina* luciferin resulted in different products. Johnson, van Schouwenburg, and

* See page 211 of their paper.

van der Burg suggested, in addition, that the two oxidations might well occur on different portions of the luciferin molecule. That is, that the molecule might have one group which might easily undergo reversible oxidation and reduction, while an entirely different group might be irreversibly oxidized with light emission.

A privately printed doctoral thesis by C. J. P. Spruit (1946) has recently appeared. This contains, among other things, the ultraviolet absorption spectra of a number of compounds with structures related to those that have been suggested for luciferin as a result of the work of Anderson (1936), Giese and Chase (1940), Chakravorty, and Ballentine (1941), van der Kerk (1942), Kluyver, van der Kerk, and van der Burg (1942), and Chase (1943). Spruit concludes that bacterial luciferin may have the following structure:



The compounds whose absorption spectra he measured include some thirty-three anthra-, naphtho- and benzoquinone and hydroquinone derivatives. Since Spruit's conclusion as to the structure of bacterial luciferin depends upon a comparison of the spectra of these known compounds with that of luciferin, the absorption spectrum of luciferin itself should be established more definitely than has so far been possible before the structure suggested by Spruit can be considered proven. The ultraviolet absorption spectra so far reported for *Cypridina* and bacterial luciferin, whether measured by direct or indirect methods, may easily have been distorted by the presence of impurities. Consequently, comparisons with the spectra of known compounds must be made with caution and a good deal of reservation.

Johnson (1947) in a comprehensive review paper on bacterial luminescence and Johnson and Eyring (1944) have called attention to the possibility that luciferin may be a prosthetic group on the protein enzyme, luciferase. Part of their evidence for this hypothesis is their demonstration that *Cypridina* luciferase solutions which have been subjected to prolonged dialysis can be made to luminesce by the addition of Na₂S₂O₄, followed by aeration of the solutions. They suggest that this prosthetic group may possibly be of flavin nature, although most of the experimental evidence available at present would appear not to favor such a structure for the luciferin molecule (Anderson and Chase, 1944; McElroy and Ballentine, 1944).

The purification of *Cypridina* luciferin by crystallization has been a goal of all workers in the chemical field of bioluminescence since luciferin was first extracted but, save for one doubtful preliminary report, no one has succeeded in obtaining crystals. Kanda (1932) reported crystallization of luciferin but his paper did not contain photographs of the crystals. The work has not been confirmed by other investigators nor have further papers on the subject been published by him.

The only crystalline derivative of luciferin so far reported is the micro-crystalline precipitate mentioned by Chakravorty and Ballentine (1941), which was obtained on treatment with hydroxylamine acetate. Here, too, no photographs are shown. When the irreversible reaction between purified luciferin and cyanide (Giese and Chase, 1940) was first observed, indicating the possible presence in luciferin of an aldehyde or keto group, an attempt was made to obtain a crystalline derivative by reaction with 2,4-dinitrophenylhydrazine. However, this was not successful. Obviously, the formation of any kind of crystalline derivative, whether capable of producing light with luciferase or not, would be valuable as a means of obtaining material of a high degree of purity for quantitative analysis, as a lead towards establishment of structure. The scarcity of the starting material, *Cypridina*, means that micro-methods, rather than the easier macro-techniques, must be used in all such work.

It may be interesting to summarize briefly some unpublished data by Dr. Harvey and myself on relative solubilities of *Cypridina* luciferin, both crude and purified, in various solvents. The amount of luciferin in solution was determined by measuring the total light available from an aliquot of the solution, on addition of luciferase, at constant pH. As had been observed earlier by Harvey, by Kanda, and by Anderson, we, too, found that great differences in the relative solubility in different solvents existed, depending upon whether the crude, dry *Cypridina* powder or the purified luciferin were used. The purified luciferin (Anderson's method), in the quantity used, was found to be completely soluble in the following solvents: methanol, ethanol, butyl alcohol, isobutyl alcohol, and propyl alcohol. Amyl alcohol, acetone, chloroform, aniline, and 0.06 *M*, pH 6.8 phosphate buffer dissolved only 50-75 per cent of the available luciferin. Ethyl ether, petroleum ether, and benzene dissolved none.

Luciferin in the form of the dry, powdered *Cypridina* material was found to be most soluble in methanol. Calling this value 100 per cent, ethanol dissolved about 16 per cent, propyl alcohol about 10 per cent, and butyl alcohol, isobutyl alcohol, amyl alcohol, acetone, chloroform, and ethyl ether dissolved practically none (0.5 per cent). Preliminary benzene extraction of the crude, powdered material in a Soxhlet caused a significant increase in the relative amounts of luciferin extractable by ethanol, butyl alcohol, and propyl alcohol. No measurable luciferin was

removed by the benzene in the preliminary treatment. The details are given in TABLE 1.

TABLE 1
RELATIVE SOLUBILITIES IN VARIOUS SOLVENTS OF PURIFIED LUCIFERIN, LUCIFERIN
IN DRY *Cypridina* POWDER, AND LUCIFERIN IN BENZENE-EXTRACTED
DRY *Cypridina* POWDER

Solvent	Relative amount of luciferin extracted		
	Purified luciferin	Untreated <i>Cypridina</i> powder	Benzene-extracted powder
Methyl alcohol	98	100	100
Ethyl alcohol	94	16	34
Butyl alcohol	100	4	12
Isobutyl alcohol	100	5	—
Propyl alcohol	98	10	24
Amyl alcohol	83	0	5
Acetone	74	1	0
Chloroform	44	3*	6*
Ethyl ether	0	0	2
Petroleum ether	0	—	—
Benzene	0	—	—
Aniline	77	—	—
Phos. buff., pH 6.8	49	—	—

* Solid fragments present in the solution.

It seems quite clear, from these results on relative solubilities, that the luciferin, as it occurs in the crude state in the gland of *Cypridina*, must be bound in some way, or else be actually different, chemically, than it is in the purified condition. This same conclusion was reached earlier by both Harvey and Kanda. If there is an actual chemical difference, this cannot be great enough to alter the structure sufficiently to preclude reaction with the enzyme, luciferase. These differences between the unpurified and purified material certainly make it evident that experimental work must be confined to only one kind of luciferin preparation: preferably, at least at this stage, to luciferin purified by Anderson's method. Otherwise, inconsistencies are bound to occur which will confuse, rather than help to clarify, the problem.

As will have become apparent, the chemical structure of the luciferin of *Cypridina* is still far from known, although a number of facts have accumulated that bear directly on the problem, and several partial structures have been postulated on the bases of these data. It seems likely that the reversible oxidation may represent a reaction analogous to the oxidation of hydroquinone. Furthermore, the redox potential of the luciferin system certainly resembles those of naturally occurring polyhydroxybenzene derivatives whose oxidized forms are unstable, as is also the case with luciferin. The irreversible reaction between purified luciferin and cyanide may indicate a free aldehyde or keto group, and the

chemical experiments of Chakravorty and Ballentine point to some sort of ketohydroxy side chain. The reaction with azide may indicate a quinone nucleus for reversibly oxidized luciferin, although this interpretation is only one of several which could be made in the light of those data. The evidence for a naphthohydroquinone structure with a ketohydroxy side chain for bacterial luciferin is probably not sufficiently conclusive to do more than indicate such a possibility. The published measurements of the ultraviolet absorption spectrum of *Cypridina* luciferin, although they, too, suggest an aromatic ring structure as the skeleton of the molecule, suffer from impurities and require remeasurement under a variety of conditions. The splitting-off of phosphate in the luminescent reaction certainly seems relevant in a consideration of structure.

A promising lead for determining the structure of luciferin may be its rather peculiar color change during oxidation. The change in spectral absorption from 435 m μ to 465 m μ on exposure to air and the subsequent loss of all visible light absorption on further exposure are rather unique properties. Any known compound which possesses such properties should be capable of transformation, by suitable chemical treatment, into luciferin. Since the luminescent reaction itself is enzyme-catalyzed, it is obvious that, in addition to the fundamental structure of the molecule, there must be one or more very specific groupings. The problem, then, once the basic structure has been found from absorption spectrum data, would involve making various derivatives by substitution of side chains, until a compound is obtained which can react with luciferase to give luminescence.

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THE FUNDAMENTAL ACTION OF PRESSURE, TEMPERATURE, AND DRUGS ON ENZYMES, AS REVEALED BY BACTERIAL LUMINESCENCE

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With any given enzyme reaction or more complex biological process, a full interpretation of the kinetics involves an understanding of the mechanisms through which temperature, hydrostatic pressure, and inhibitors of various kinds influence the observed rate. While the theory of equilibria in regard to temperature, pressure, and concentration of reactants has been known for some years, it is scarcely a decade since the rational and precise theory for change became available, in the theory of absolute reaction rates^{1, 2} for a rate process such as that of a simple chemical reaction. Inasmuch as the same theory undoubtedly holds as well for each of the individual reactions which collectively lead to any biological phenomenon, the problem is to see how it may be applied to single reactions in the midst of many, and to what extent it may account, quantitatively, for the process as a whole. In some cases, for example with an extracted and purified enzyme system, the kinetics might be expected to be relatively uncomplicated, although simultaneous reactions involving the same molecule may influence the over-all rate of the measured process. In living cells, on the other hand, a series of systems is generally concerned, and under various conditions one or more systems may be largely rate-determining, in each case influenced in their activity by simultaneous equilibria and rate processes. When a single system remains largely limiting, however, it is reasonable to believe that even complex processes might be analyzed, approximately, in the manner of a single reaction.

For investigating the problem at hand, luminescence possesses distinct, and in certain respects unique advantages. In the first place, the intensity of the light is evidently proportional to the reaction velocity of the "luciferase" with "luciferin," as shown by kinetic studies with crude as well as with partially purified extracts of *Cypridina*.³ Although luminescent extracts have not as yet been obtained from bacteria, spectroscopic and other lines of evidence indicate that the light-emitting system is not fundamentally different in the two cases. Inasmuch as the intensity of luminescence may be easily recorded by means of a photoelectric cell or other devices,⁴ the rate of reaction, in relative units, can be determined with considerable accuracy for a given instant. Likewise, the course of more or less rapidly changing reaction rates may be followed during very brief as well as over longer periods of time.

In non-reproducing bacteria, under favorable physiological conditions, luminescence is in a steady state, with constant intensity. In extracts of *Cypridina*, the reaction is first order with respect to both the concentration of dihydroluciferin and of active luciferase, and the luminescent oxidation is accompanied by the destruction of a large part of the luciferin.^{3, 5} In bacteria, if any considerable destruction of the luciferin accompanies the light-emitting oxidation, the luciferin must be formed from some precursor at the same rate, in order for a uniform intensity to be maintained. Since excited molecules are generally stabilized by radiating, whereas excited molecules which do not radiate are apt to be destroyed, repeated oxidation and reduction of the same luciferin molecules very likely occurs, with perhaps much less of the destructive reaction in living cells than in extracts. In any case, the uniform luminescence intensity of bacteria indicates that the concentration of the reactants, luciferin and luciferase, in effect remains constant with time. Thus, the intensity (I) of the light will be proportional to the amount of active luciferase (A_n) times the amount of reduced luciferin (LH_2) times the specific reaction rate constant, k , times some proportionality constant, b :

$$I = bk(A_n)(LH_2). \quad (1)$$

While it is apparent that several equilibria and rate processes precede actual light emission⁶ the evidence from flow-method studies with *Cypridina* extracts⁷ indicates that EQUATION 1 represents the slowest reaction. A scheme of consecutive reactions consistent with the known facts concerning luminescence in bacteria and in extracts is given in TABLE 1. Excited luciferin is designated by L^* , and destroyed luciferin by L_1 .

TABLE 1

Reactions with luciferase		Additional reactions that occur with and without luciferase	
(1)	$AL + XH_2 \rightleftharpoons ALH_2 + X$	(1')	$L + XH_2 \rightleftharpoons LH_2 + X$
(2)	$A + LH_2 \rightleftharpoons ALH_2$		
(3)	$ALH_2 + O_2 \rightleftharpoons ALH + HO_2$	(3')	$LH_2 + O_2 \rightleftharpoons LH + HO_2$
(4)	$ALH \rightleftharpoons AL^- + H^+$	(4')	$LH \rightleftharpoons L^- + H^+$
(5) alpha	$AL^- + LH \rightarrow AL^* + LH^- \rightarrow AL$	(5') alpha	$L^- + LH \rightarrow L^* + LH^- \rightarrow L +$
	$+ LH^- + h\nu$		$LH^- + h\nu$
(5) beta	$AL^- + LH \rightarrow AL + LH^-$	(5') beta	$L^- + LH \rightarrow L + LH^-$
(5) gamma	$AL^- + LH \rightarrow AL_1 + LH^-$	(5') gamma	$L^- + LH \rightarrow L_1$
(6)	$ALH + O_2 \rightarrow AL + HO_2$	(6')	$LH + O_2 \rightarrow L + HO_2$
(7)	$AL + O_2 \rightarrow AL_1$	(7')	$L + O_2 \rightarrow L_1$

In this scheme, the transfer of an electron between two semi-quinone forms of the luciferin leads to excitation, followed by radiation. The luciferin is presumed to be the radiating molecule, because of the cor-

respondence between the absorption spectrum of luciferin and the emission spectrum of the luminescent reaction,^{8, 9} together with the fact that luciferin emits light in 95 per cent alcohol at 70° C.,¹⁰ a condition under which the enzyme might be expected to be inactive.

Thus, the over-all process of luminescence may be limited in various ways, even though reaction 2 ordinarily remains the slowest of the series. At very low oxygen tensions, for example, reaction 3 becomes limiting. In acid pH, the amount of ALH decreases and, to this extent, reaction 4 may be considered limiting. In alkaline pH, the amount of LH may be considered limiting, etc. All these reactions, however, are fast in comparison with reaction 2, even though alterations in the steady state concentration of subsequent reactants, *e.g.*, by changes in pH, influence the level of luminescence intensity in a corresponding manner. At optimum pH, with oxygen not limiting, and with excess of glucose, the over-all rate is determined by the specific reaction rate constant, the amount of luciferin, and the amount of active luciferase, as given in EQUATION 1.

A second, unusual advantage of luminescence in analyzing the kinetics of over-all processes that occur in living cells is that the enzyme system concerned is not preceded by a considerable number of other systems engaged in a stepwise hydrogen or electron transfer. The evidence that this is so derives from the facts that (a) glucose added to washed cells immediately results in large increases in luminescence, showing that some of the hydrogen from glucose is transferred *via* the luciferin-luciferase system, with light emission;^{11, 12, 13} and (b) the wavelength of maximum intensity corresponds to a transition with $\Delta F^\circ = 60,700$ calories, as compared with the average ΔF° of 57,340 calories for two hydrogens in the oxidation of glucose.⁶ Thus, in luminescence, some fraction of the hydrogens from glucose goes almost directly to oxygen, by way of the luminescent system, and the energy is released through visible radiation.

Finally, since luminescence may be studied both in living bacterial cells and in a corresponding system extracted and partially purified from *Cypridina*, it is possible to ascertain whether inhibitors which influence the intensity of the light in cells also act, at equivalent concentrations, on the system *in vitro*. Moreover, by measuring the effect of a given inhibitor on the total light in extracts, it is possible to distinguish between those which, like certain ions¹⁴ quench luminescence, and those, such as urethane or sulfanilamide,¹⁵ which retard the rate of the enzyme reaction without affecting the total light produced.

In order to account for the reversible effects of temperature and hydrostatic pressure on bacterial luminescence, it is necessary to modify EQUATION 1 to include an equilibrium between active and inactive forms of one of the reactants.^{16, 17} Since this equilibrium, which rapidly becomes a conspicuous limiting factor with rise in temperature beyond the normal optimum, has been found to be characterized by the high heat

and entropy typical of protein denaturation, it evidently concerns the enzyme.^{18, 19, 20} Letting K_1 represent the equilibrium constant between active (A_n), and inactive or reversibly denatured (A_d) forms of the enzyme, and A_0 the total of $(A_n) + (A_d)$, EQUATION 1 becomes

$$I_1 = \frac{bk(LH_2)(A_0)}{1 + K_1}, \quad (2)$$

which, by definition of the equilibrium constant K_1 and of the rate constant k in accordance with the theory of Absolute Reaction Rates^{1, 2} may be written:

$$\begin{aligned} I_1 &= \frac{b \kappa \frac{kT}{h} e^{-\frac{\Delta F^\ddagger}{RT}} (LH_2)(A_0)}{1 + e^{-\frac{\Delta F_1}{RT}}} \\ &= \frac{b \kappa \frac{kT}{h} e^{-\frac{\Delta H^\ddagger}{RT}} e^{\frac{\Delta S^\ddagger}{R}} (LH_2)(A_0)}{1 + e^{-\frac{\Delta H_1}{RT}} e^{\frac{\Delta S_1}{R}}} \\ &= \frac{b \kappa \frac{kT}{h} e^{-\frac{\Delta E^\ddagger}{RT}} e^{-\frac{p}{RT}} e^{\frac{\Delta V^\ddagger}{RT}} e^{\frac{\Delta S^\ddagger}{R}} (LH_2)(A_0)}{1 + e^{-\frac{\Delta E_1}{RT}} e^{-\frac{p}{RT}} e^{\frac{\Delta V_1}{RT}} e^{\frac{\Delta S_1}{R}}}. \end{aligned} \quad (3)$$

EQUATION 3 contains some unknown quantities, (LH_2) , (A_0) , and ΔS^\ddagger , which cannot be readily determined. Under chosen conditions, however, where luminescence of fully aerated, non-reproducing cells is constant with time, these quantities may be assumed essentially constant. The same applies to κ , the transmission coefficient, k , the Boltzman constant, h , Planck's constant, and b , the proportionality constant. Consequently, they may be included in a single constant, c , and EQUATION 3 then becomes

$$I_1 = \frac{c T e^{-\frac{\Delta H^\ddagger}{RT}}}{1 + e^{-\frac{\Delta H_1}{RT}} e^{\frac{\Delta S_1}{R}}} = \frac{c' T e^{-\frac{\Delta E^\ddagger}{RT}} e^{-\frac{p}{RT}} e^{\frac{\Delta V^\ddagger}{RT}}}{1 + e^{-\frac{\Delta E_1}{RT}} e^{-\frac{p}{RT}} e^{\frac{\Delta V_1}{RT}} e^{\frac{\Delta S_1}{R}}}. \quad (4)$$

EQUATIONS 3 and 4 show that the over-all intensity of luminescence, under optimal physiological conditions of pH, oxygen, salt concentration, etc., will be determined by the influence of temperature on two reactions involving the same molecule, namely, the rate of the catalytic reaction and the inactivation equilibrium of the enzyme, respectively.

The values of the constants in EQUATION 4 may be estimated from experiments in which I is measured at different temperatures and pressures. Thus, the apparent ΔH^\ddagger , which at normal pressure is indistinguishable from " μ " of the Arrhenius equation, is computed from the slope of the line relating $\ln I$ and $\frac{1}{T}$ in the low temperature range where the value of K_1 is negligible. ΔH_1 is obtained from the slope of the decreasing I with increasing T at temperatures above the optimum, and adding to this the value of ΔH^\ddagger , disregarding signs. ΔV^\ddagger may be determined from the slope of the line relating $\ln I$ to pressure (p) in atmospheres at low temperatures. ΔV_1 is obtained from a similar plot at high temperatures, ΔE from the relation, $\Delta H = \Delta E + p\Delta V$, and ΔS from the relation

$$K = e^{-\frac{\Delta H}{RT}} e^{\frac{\Delta S}{R}} = e^{-\frac{\Delta E}{RT}} e^{-p\frac{\Delta V}{RT}} e^{\frac{\Delta S}{R}}.$$

These constants then make it possible to calculate, using EQUATION 4, the intensity of luminescence with some accuracy at various temperatures and pressures, as shown (FIGURE 1) by Eyring and Magee.¹⁹ In this

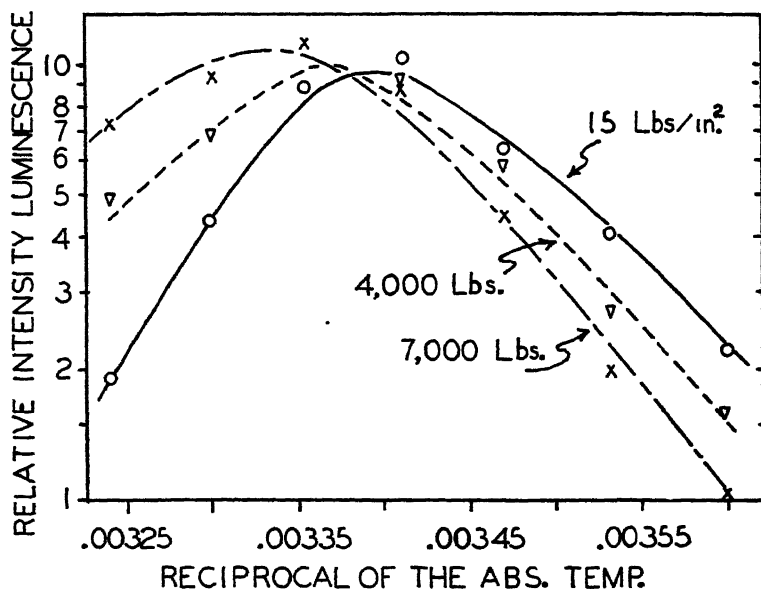


FIGURE 1. Relation between the intensity of luminescence (*Photobacterium phosphoreum*) and temperature, under normal and increased hydrostatic pressures of 4,000 and 7,000 lbs./sq. in., respectively. The solid lines are curves calculated by Eyring and Magee¹⁹ as described in the text; the points represent data from experiments of Brown, Johnson, and Marsland.¹⁷ Logarithmic scale on the ordinate.

case, ΔV^\ddagger amounts to 50 cc. per mole at 0° C, and ΔV_1 to 64 cc. at 35° C, but a temperature dependence of these constants has been taken into account. The constants employed are as follows: $\Delta E^\ddagger = 17,220$ calories, $\Delta V^\ddagger = 546.4 - 1.813T$ cc., $\Delta E_1 = 55,260$ calories, $\Delta S_1 = 184$ Entropy units, $\Delta V_1 = -922.8 + 3.206T$ cc.

The difference in the values of the constants for the luciferin-luciferase reaction and for the inactivation equilibrium of luciferase, respectively, are largely responsible for the optimal temperature of luminescence at a given pressure, and likewise for an optimal pressure at a given temperature. The apparent activation energy of 17,220 calories at atmospheric pressure is similar to, or not very different from, those familiarly associated with enzyme reactions and more complex biological processes as a whole. The heat and entropy of the inactivation equilibrium, as pointed out above, are typical of protein denaturation. The volume changes of activation and of reaction, respectively, are of particular interest, since both are high and show that in each case the reaction involves a very large molecule. These volume changes indicate that fairly drastic alterations in the structure of the molecule take place both in the formation of the activated complex and in the reversible inactivation. They probably indicate considerable unfolding of the protein from a somewhat globular to a more fibrous form. Thus, in the process of catalysis, it would appear necessary for the enzyme to change its configuration to fit the substrate, although such a change would not be necessary if the active or combining groups of the enzyme are at the surface, which appears to be the situation with invertase.²¹

Turning now to the action of inhibitors which combine reversibly with the luminescent system, it is evident from EQUATION 2 that the equilibrium through which the system is inactivated may be established either independently of the denaturation equilibrium (Type I) or in relation to it (Type II). The former mechanism has the likeness of a combination of the inhibitor with a prosthetic group of the enzyme, or possibly the luciferin. In this case, kinetic data alone are not sufficient to distinguish between the enzyme and substrate as site of action.¹⁸ Type II represents a combination of the inhibitor with bonds which are involved in the denaturation equilibrium. Although Types I and II cannot be distinguished experimentally on the basis of the relation between concentration of inhibitor and the amount of inhibition observed, other conditions remaining constant, they may, in general, be expected to behave in different ways as pressure and temperature are varied. Thus, in Type I, letting K_2 represent the equilibrium constant, independent of K_1 , pressure would be expected to have only slight influence, while a rise in temperature should result in a decrease in per cent inhibition, as the enzyme-inhibitor complex is dissociated and K_2 becomes smaller. Since the inhibition at a given drug concentration is less at lower temperatures, an

increase in apparent activation energy of the luminescent reaction occurs in the presence of the drug, and the temperature of maximum luminescence is slightly increased. On the other hand, in Type II, with equilibrium constant K_s , because of its dependence on K_1 , which is characterized by a large volume change of reaction, a pronounced effect of pressure on the inhibition would be anticipated. Thus, at temperatures near the normal optimum, moderate inhibitions by alcohol can be completely reversed by hydrostatic pressure,⁶ as illustrated in FIGURE 2. The influence

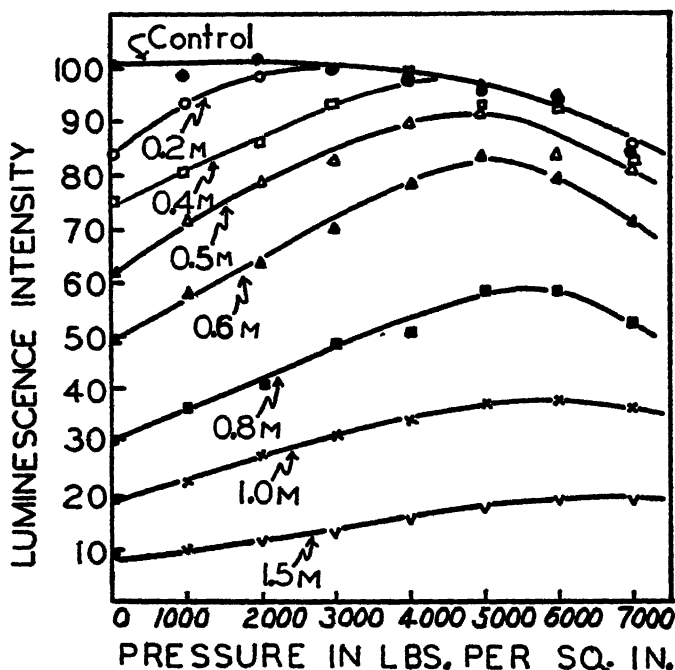


FIGURE 2. The relation between hydrostatic pressure and the amount of inhibition of luminescence at 17.5° C caused by the various concentrations of alcohol indicated on the figure.⁶ (*Photobacterium phosphoreum*.)

of pressure on the inhibition caused by various drugs, including representatives of both types, is illustrated in FIGURE 3.²² As for temperature, the influence in this type depends on both K_1 and K_s , that is: although the enzyme-inhibitor complex dissociates with rise in temperature, the bonds with which the inhibitor combines may be more available; K_1 increases and K_s decreases as the temperature is raised, and the net result depends on K_1 times K_s . In the cases studied, the per cent inhibition increases with rise in temperature, resulting in a lowering of the apparent activation energy, a decrease in the observed heat of denaturation, and a

somewhat lower temperature of maximum luminescence, in the presence of a given drug concentration.

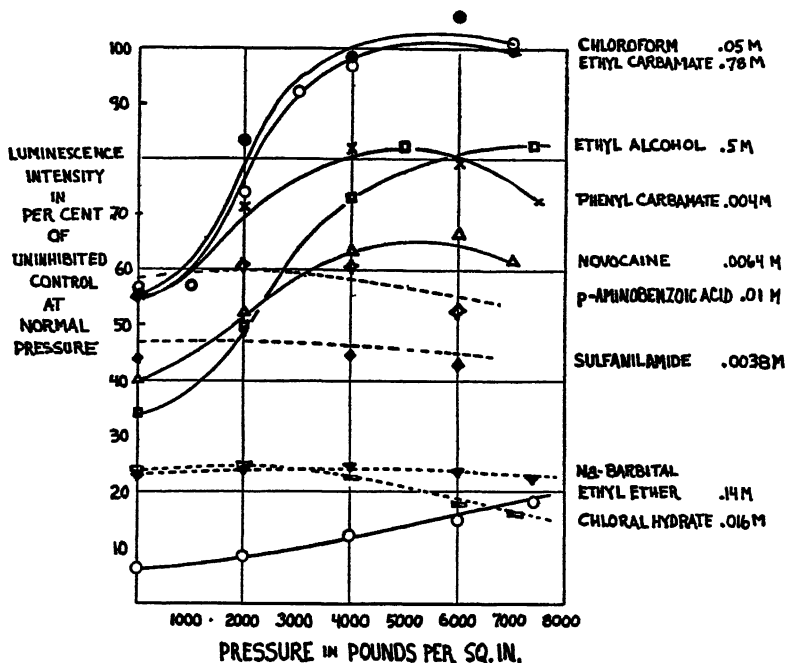


FIGURE 3. The relation between hydrostatic pressure and the amount of inhibition of luminescence caused by a given concentration of various drugs, at 17 to 18° C. The intensity of the drug-free control at atmospheric pressure has arbitrarily been taken as 100, and the other points computed in relation to it. (*Photobacterium phosphoreum*.²²)

The relation between luminescence intensity and temperature, without the addition of drugs, and in the presence of a single concentration of sulfanilamide and urethane, respectively, is illustrated in FIGURE 4.⁶

These respective mechanisms may be formulated, and expressions obtained for arriving at the constants, K_s and K_3 , from the data of experiments. Thus, letting I_s represent the observed luminescence intensity in the presence of a given concentration of inhibitor, we have, for Type I,

$$I_s = \frac{bk(LH_2)(A_0)}{1 + K_1 + K_2Xr + K_1K_2Xr}, \quad (5)$$

in which X represents the molar concentration of inhibitor, and r the ratio between the inhibitor and enzyme molecules in the complex formed. Dividing EQUATION 2 by EQUATION 5 and simplifying,

$$\left(\frac{I_1}{I_s} - 1\right) = K_2Xr. \quad (6)$$

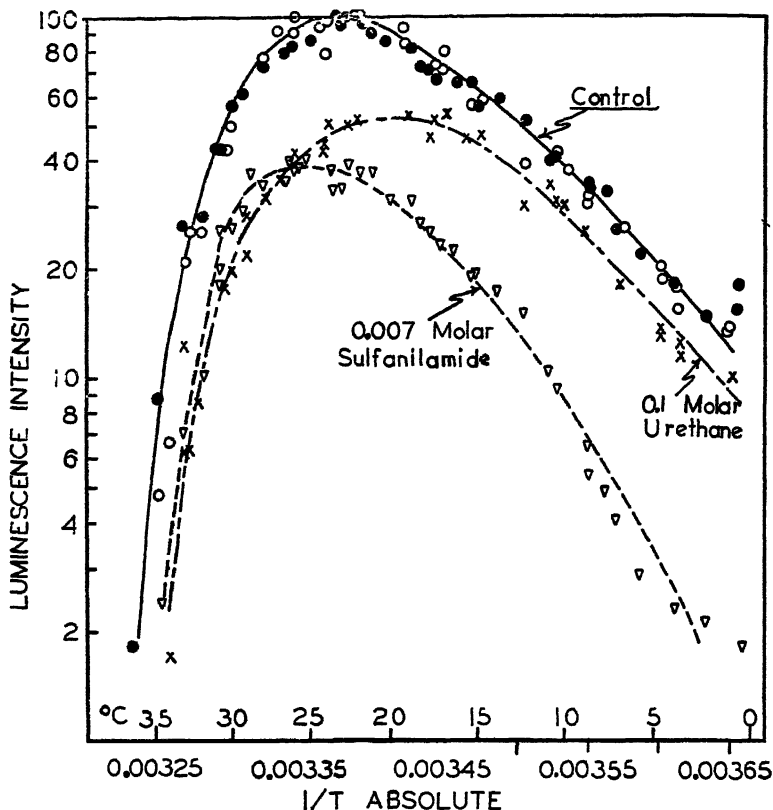


FIGURE 4. Luminescence intensity of *Photobacterium phosphoreum* in relation to temperature in a control and in corresponding suspensions containing 0.007 molar sulfanilamide and 0.1 molar urethane, respectively. The solid and hollow circles for the control are from repeated experiments. The data are replotted from Johnson, Eyring, Steblay, et al., 1945.⁶

Similarly, for Type II,

$$I_2 = \frac{bk(LH_2)(A_0)}{1 + K_1 + K_1K_sU^s}, \quad (7)$$

in which U represents the molar concentration of inhibitor, and s the ratio of drug-enzyme molecules in the complex. Dividing EQUATION 2 by EQUATION 7 and simplifying,

$$\left(\frac{I_1}{I_2} - 1\right) = \frac{K_1K_sU^s}{1 + K_1}, \text{ or } \left[\left(\frac{I_1}{I_2} - 1\right) \left(1 + \frac{1}{K_1}\right)\right] = K_sU^s. \quad (8)$$

With the aid of EQUATIONS 6 and 8, the two mechanisms may be distinguished. In either case, a plot of $\ln \left(\frac{I_1}{I_2} - 1\right)$ against \ln molar concentration of inhibitor at constant temperature and pressure, yields

straight lines whose slope gives the average number of inhibitor molecules combining with each enzyme molecule (FIGURE 5), unless, of course,

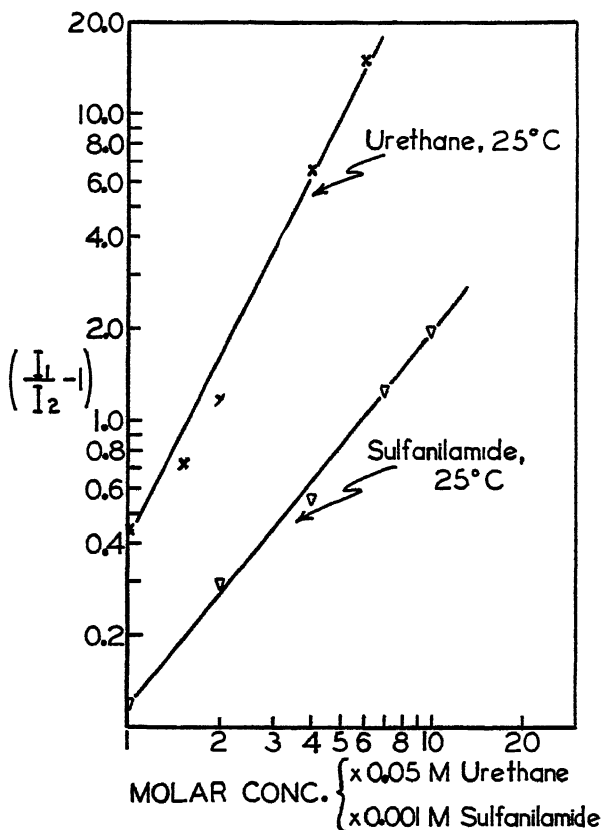


FIGURE 5. Relation between the concentration of sulfanilamide and urethane, respectively, and the amount of inhibition of bacterial luminescence, (*Photobacterium phosphoreum*) at 25° C., plotted in the manner discussed in the text.⁹ The slope of the line for sulfanilamide is 1.2; for urethane, 2.0. With urethane, the slope increases with temperature, although with sulfanilamide there is little change.

more than one system is affected or additional reactions are involved, so that the action is more complicated than the theory takes into account.

When, on the other hand, $\ln \left(\frac{I_1}{I_2} = 1 \right)$ is plotted against the reciprocal of the absolute temperature, a straight line over a wide range of temperatures is obtained for Type I, but not for Type II (FIGURE 6). From the slope of the straight line, the heat of reaction, ΔH_2 , of the equilibrium may be computed. If the mechanism is Type II, a straight line,

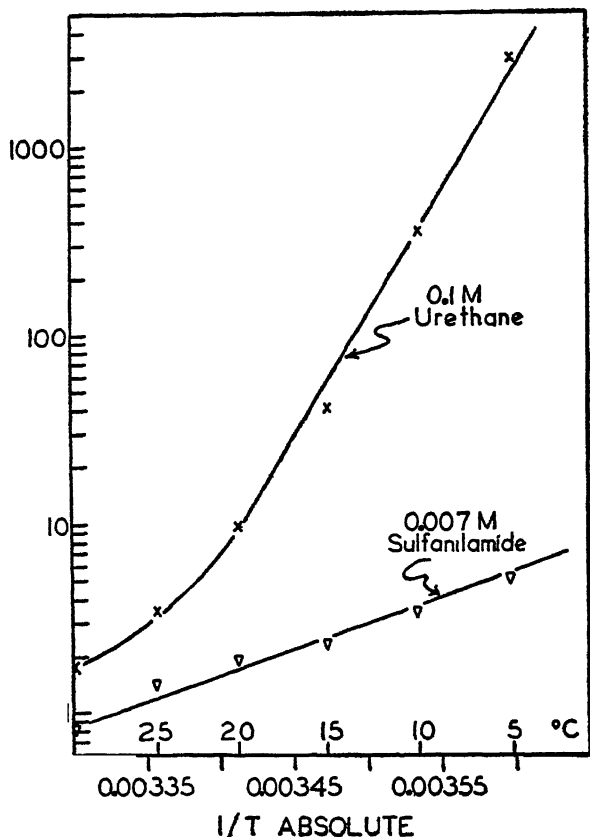


FIGURE 6. Analysis of the curves shown in FIGURE 4, according to the formulations given in the text. The luminescence intensity of the sulfanilamide-containing suspension (I_2) in relation to that of the control (I_1) is plotted on the logarithmic scale of the ordinate as $\left(\frac{I_1}{I_2} - 1\right)$ against the reciprocal of the absolute temperature on the abscissa. The slope indicates 12,500 calories as the heat of reaction in the sulfanilamide-enzyme equilibrium. The values of the expression $\left(\frac{I_1}{I_2} - 1\right)$ for the urethane curve in FIGURE 4 in relation to the control have been multiplied by $\left(1 + \frac{1}{K_1}\right)$, and the product plotted on the ordinate for different values of $1/T$. In this case, the slope of the line, through the lower temperature range, indicates a heat of reaction of approximately 60,000 calories in the urethane-enzyme equilibrium.

whose slope depends on the heat of reaction, ΔH_s , results when $\ln \left[\left(\frac{I_1}{I_2} - 1 \right) \left(1 + \frac{1}{K_1} \right) \right]$ is plotted against $\frac{1}{T}$ (FIGURE 6). In the cases studied, however, the relation frequently departs from linearity, in the direction of too high an inhibition at temperatures beyond the normal optimum. Inasmuch as such deviations have been found to be more

marked with increasing concentration of the drug, and since Type II inhibitors have been found to promote an irreversible as well as a reversible denaturation of the luminescent system,⁶ it appears likely that, under these conditions, the irreversible effects are added to the reversible ones. Only the latter are taken into account by the above formulation.

In a purely diagrammatic way, the influence of temperature, pressure, and inhibitors of the two types discussed above may be illustrated as shown in FIGURE 7. Thus, at given concentrations of substrate and enzyme,

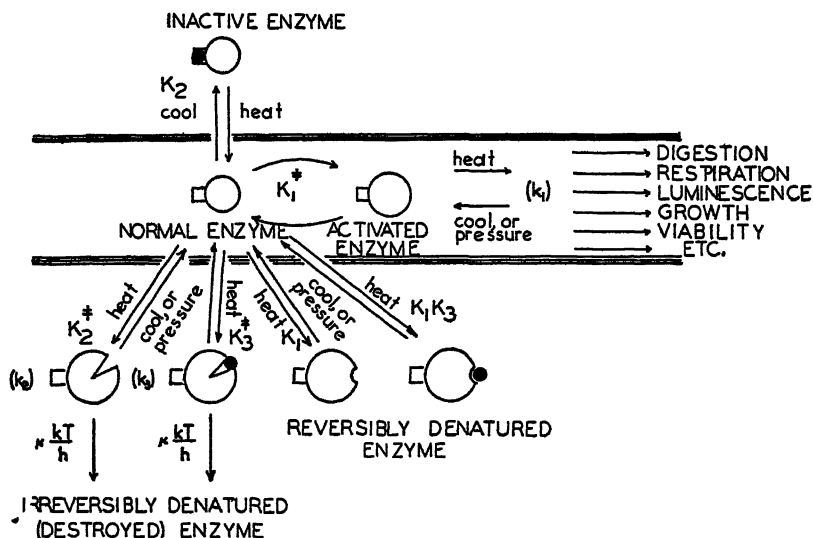


FIGURE 7. Schematic diagram to illustrate the control of the luminescent oxidation (k_1) in relation to temperature, hydrostatic pressure, reversible Type I inhibitors (K_2), reversible Type II inhibitors (K_3), the reversible temperature inactivation of the enzyme (K_1), irreversible thermal destruction (k_2), and irreversible destruction promoted by Type II inhibitors (k_3), as discussed in the text.

the rate of the reaction in a single system, or in a complex process consistently limited largely by a single system, will be accelerated by a rise in temperature, and retarded by an increase in pressure, in a manner quantitatively determined by the specific reaction rate constant, k_1 . The extent to which the rate increases for a given rise in temperature depends on the heat of activation, ΔH_1^\ddagger , in the equilibrium, K_1^\ddagger , between the reactants and the activated complex, which, once formed, decomposes with the universal frequency, $\frac{kT}{h}$, characteristic of all chemical reactions.

Likewise, the amount of the pressure effect depends on the volume change of activation, ΔV_1^\ddagger , in the same equilibrium constant, K_1^\ddagger . As long as the enzyme molecules remain within the active channel, enclosed

by the parallel lines (FIGURE 7), while the substrate concentration also is maintained at a constant, steady-state level and other factors remain the same, the reaction rate depends only on the value of k_1 .

The amount of active enzyme, however, may be altered by one or more reactions. In the first place, as indicated above, it is apparent that the enzyme normally exists in equilibrium (K_1) between active and inactive forms. Because of the high heat and entropy of this reaction, the value of K_1 at temperatures below the optimum is so small as to be negligible. In the region of the optimum and above, K_1 increases rapidly with rise in temperature, causing the proportion of inactive molecules to increase to a greater extent, by a given increment in temperature, than the activation process in the enzyme reaction is accelerated: *i.e.*, K_1 is more strongly influenced by temperature than k_1 . As a result, the observed rate goes through a maximum. Similarly with pressure: the inactivation proceeds with a large volume increase and, as a consequence, K_1 is decreased with rise in pressure and is more markedly affected than k_1 , other factors remaining the same.

In addition to the reversible inactivation, with an equilibrium constant indicative of a protein denaturation equilibrium, a rate process of thermal destruction also takes place. In luminescence, the latter reaction has an even higher activation heat and entropy than the ΔH and ΔS of the reversible denaturation (FIGURE 8).⁶ It is for this reason that the reversible denaturation of the luminescent enzyme can be so readily observed experimentally. The thermal destruction also proceeds with a large volume increase of activation, and consequently is markedly retarded by pressure at a given temperature (FIGURE 9).⁶

Both the reversible and irreversible denaturations are furthered by the addition of inhibitors of Type II, such as urethane or alcohol, apparently by the combination of these agents with bonds made available in the denaturation reaction. Both are opposed by hydrostatic pressure or by cooling.

By a mechanism different from those just described, certain inhibitors (Type I) enter into an equilibrium, K_2 , causing an inactivation of the enzyme independently of the denaturation. The combination apparently occurs equally with the active and reversibly denatured forms and does not itself lead to an alteration in structure of the protein accompanied by a large volume change. Consequently, pressure has little influence. On the other hand, the enzyme-inhibitor complex will, in general, be dissociated by a rise in temperature, so that the fraction of inactive molecules will be less at higher than at lower temperatures, and the per cent inhibition correspondingly smaller.

The generality of the mechanisms, as shown in FIGURE 7, to a large extent awaits justification by further experiments, inasmuch as data sufficient for analysis on the basis of the theory described are not widespread,

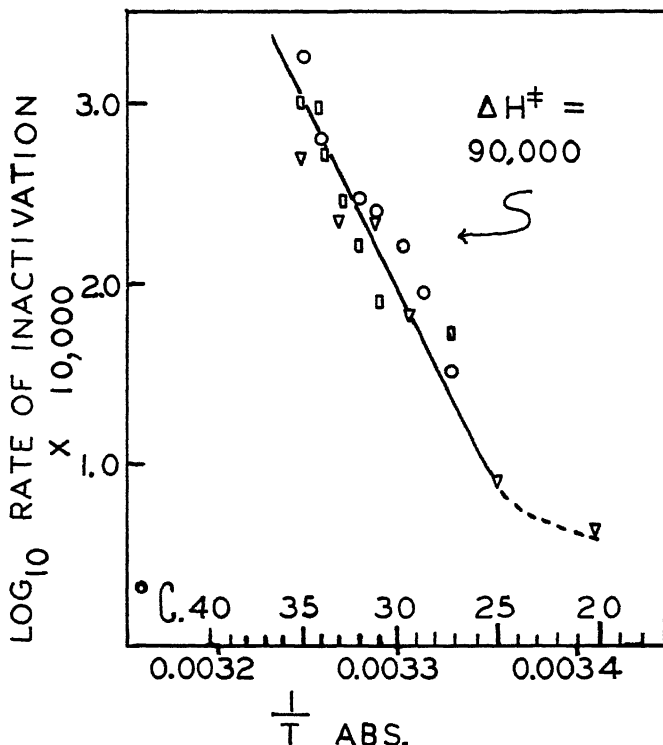


FIGURE 8. Temperature analysis of the rate of thermal destruction of the luminescent system in bacteria (*Photobacterium phosphoreum*). The points are from three repeated experiments. The slope of the line indicates an apparent activation energy of some 90,000 calories.⁶ Semi-log scale.

particularly with respect to the action of hydrostatic pressure. Moreover, previous to the studies in connection with luminescence, the possibility that protein denaturation might be opposed by hydrostatic pressure had apparently not been taken into account. The opposite effect has been known for some years, *viz.*, that very high pressures, of the order of 10,000 atmospheres, at room temperature denature proteins, kill microorganisms, and inactivate enzymes, viruses, antibodies, bacteriophage, etc. (*cf.* reviews by Macheboeuf and Basset²³ (1934), Cattell²⁴ (1936), and Bridgman²⁵ (1946)). It is perhaps worth while, therefore, briefly to consider some of the available evidence with respect to other systems than luminescence.

First of all, it is apparent that the reactions by which proteins are denatured at room temperature under very high pressures are not identical with the reactions which take place at relatively high temperatures (or at lower temperatures in the presence of certain drugs such as alcohol) and which are opposed by moderate pressures, of the order of 500 atmospheres.

The fact that the lower pressures may greatly retard the denaturation of a highly purified protein, human serum globulin, at 65° C. has recently been demonstrated.²⁶ Small concentrations of ethyl alcohol accelerate

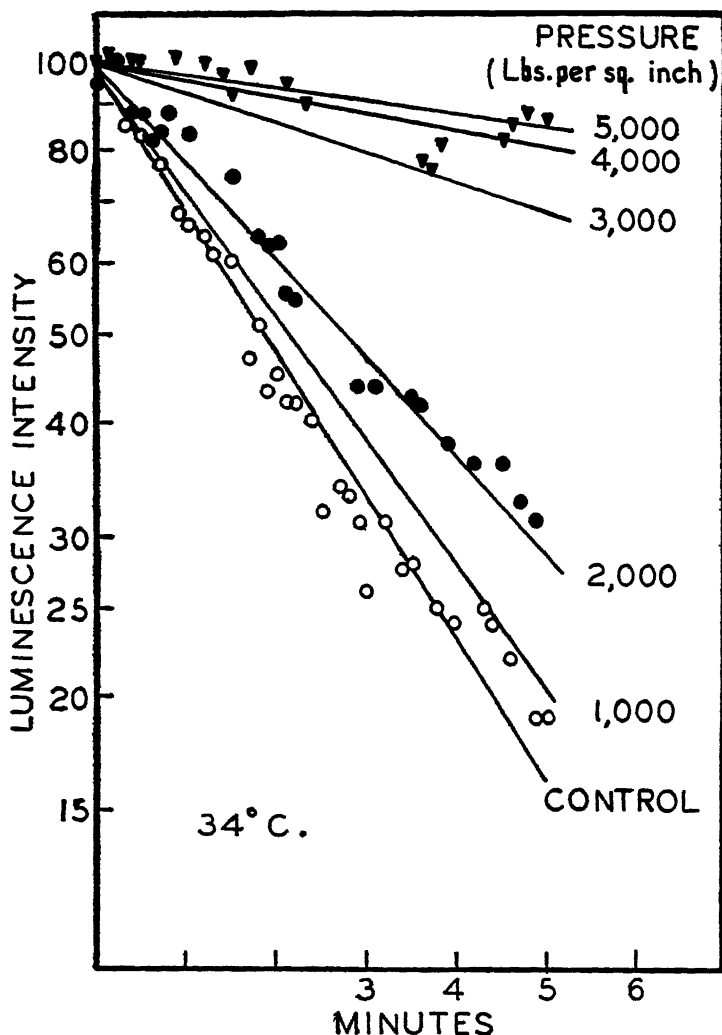


FIGURE 9. Influence of hydrostatic pressure in retarding the thermal destruction of the luminescent system at 32° C.⁶

the precipitation, while pressure retards it, with alcohol as well as without it (FIGURE 10). The rate is higher than first order. Preliminary estimates of the volume change indicate a volume increase of activation of about

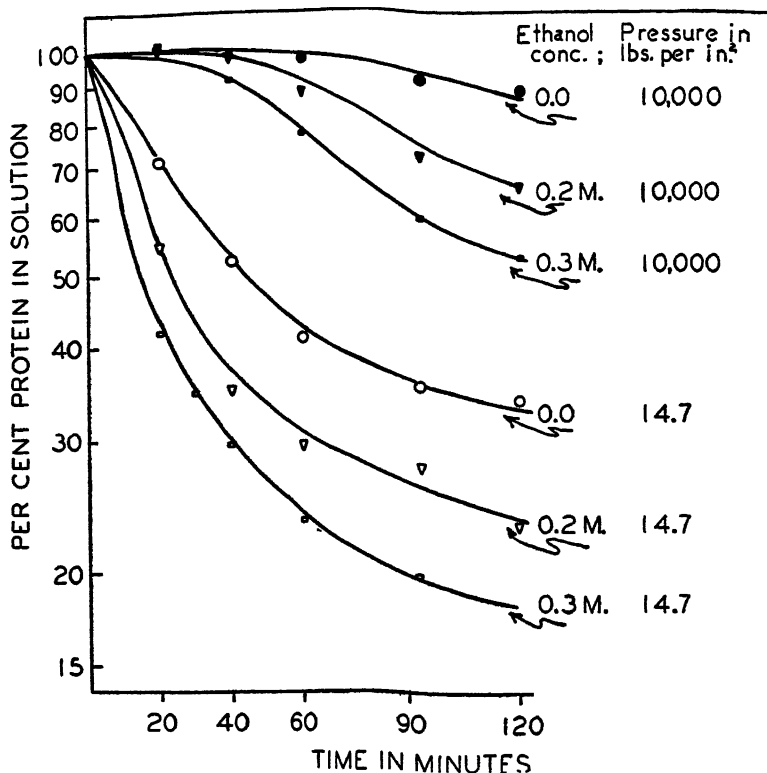


FIGURE 10. The rate of precipitation of highly purified human serum globulin at 65° C. in relation to hydrostatic pressure and small concentrations of ethyl alcohol.²⁸ Semi-log scale.

100 cc. per mole. The denaturation of specific antitoxic activity at the same temperature is also opposed by a pressure of 680 atmospheres.²⁷ Furthermore, specific precipitation of rabbit immune serum by a simple trihaptenic dye antigen, at room temperature, is greatly retarded under pressure of 680 atmospheres, indicating a large volume increase, of some 50 cc. per mole according to available data, in the reaction involving the antibody molecules.²⁵

In regard to extracted enzyme and sol-gel systems, Marsland and Brown²⁹ have shown that the sol-gel equilibrium of rabbit myosin is characterized by a volume change of 120 cc. per mole. This is especially interesting, since sol-gel reactions influencing intracellular processes involving protoplasmic streaming, *e.g.* cyclosis, amoeboid motion, cleavage of animal cells, etc.,³⁰ are also accompanied by large molecular volume changes, of the order of 102 cc. per mole.²⁹ The rate of various extracted enzyme systems, *e.g.*, lipase, pepsin, and pancreatic proteinases (Benthaus,

1942²¹; *cf.* also Deuticke and Harren, 1938³²), at room temperature, is reversibly retarded by pressures up to 1,500 atmospheres. As yet, sufficient data do not exist for a satisfactory analysis of the volume changes in these enzyme reactions. Thus, further studies should yield interesting results. Moreover, it might be expected that, under conditions favoring a reversible denaturation of the enzyme, an increase in the net activity of the system under pressure would be encountered, as in luminescence.

The activity of invertase or of diastatic enzymes is apparently not greatly retarded under moderate pressure at room temperature (Regnard, 1884³³) and may even be increased.^{21, 31} Recent studies²¹ have shown that the increase in rate of sucrose inversion by invertase is most marked under conditions of partial inactivation of the enzyme, such as high temperatures at optimum pH, or at lower temperatures in relatively alkaline or acid solution (FIGURE 11). Analysis of the data indicated

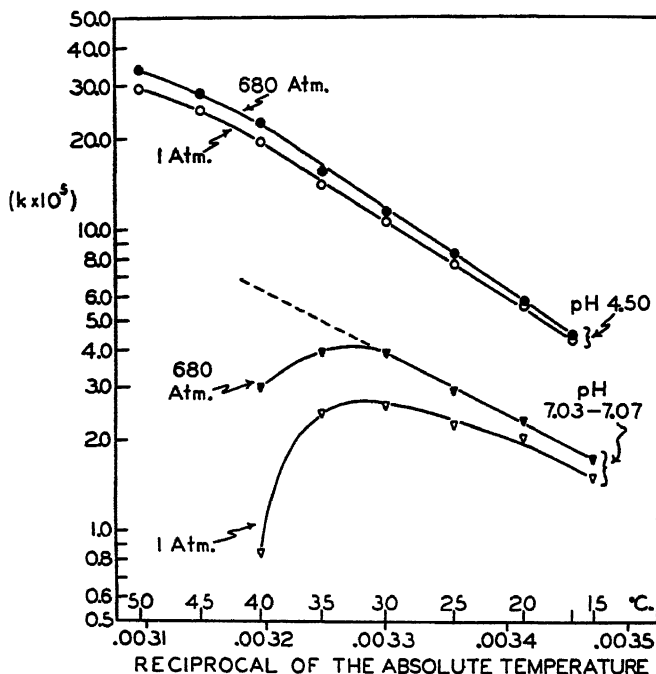


FIGURE 11. The rate, during the logarithmic period, of inversion of 10 per cent sucrose by invertase, in relation to temperature and hydrostatic pressure, at pH 4.5 and pH 7.03-7.07, respectively.²⁴

that at pH 7.05 and 35 or 40° C., the enzyme undergoes a reversible denaturation with a volume increase of about 69 cc. per mole.

In living cells, a number of phenomena, such as the tension of auricle muscle at room temperature, increase in intensity under pressures up to

about 400 atmospheres, and then decrease as the pressure is raised up to less than 1,000 atmospheres.³⁴ The similarity in effects of pressure on these processes and on luminescence suggests that corresponding mechanisms are involved. In still other complex phenomena, including the rates of microbial growth and disinfection, a reversible inhibition by pressure has been noted.³⁵ A greater multiplicity of reactions are, no doubt, concerned in bringing about the measured result in all these cases, and the analysis, therefore, is more difficult than in luminescence.

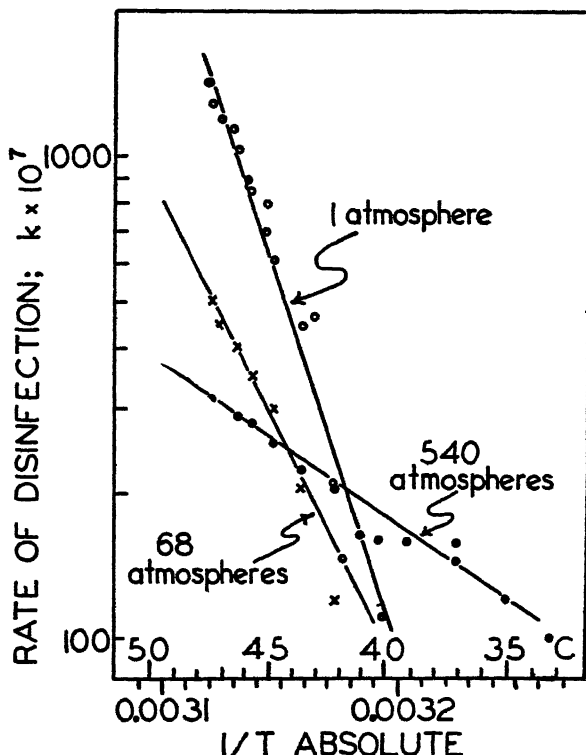


FIGURE 12. The rate of disinfection of non-proliferating cells of *Escherichia coli* in relation to temperature, under normal and increased hydrostatic pressures of 1,000 and 5,000 pounds per square inch, respectively.³⁵

FIGURES 12 and 13 illustrate some of the data pertaining to growth and disinfection.

The relation between hydrostatic pressure and amount of inhibition caused by a given concentration of a drug has apparently been studied only in connection with bacterial luminescence,^{6, 22, 36} and to a lesser extent, bacterial growth and disinfection.³⁵ Although the influence of

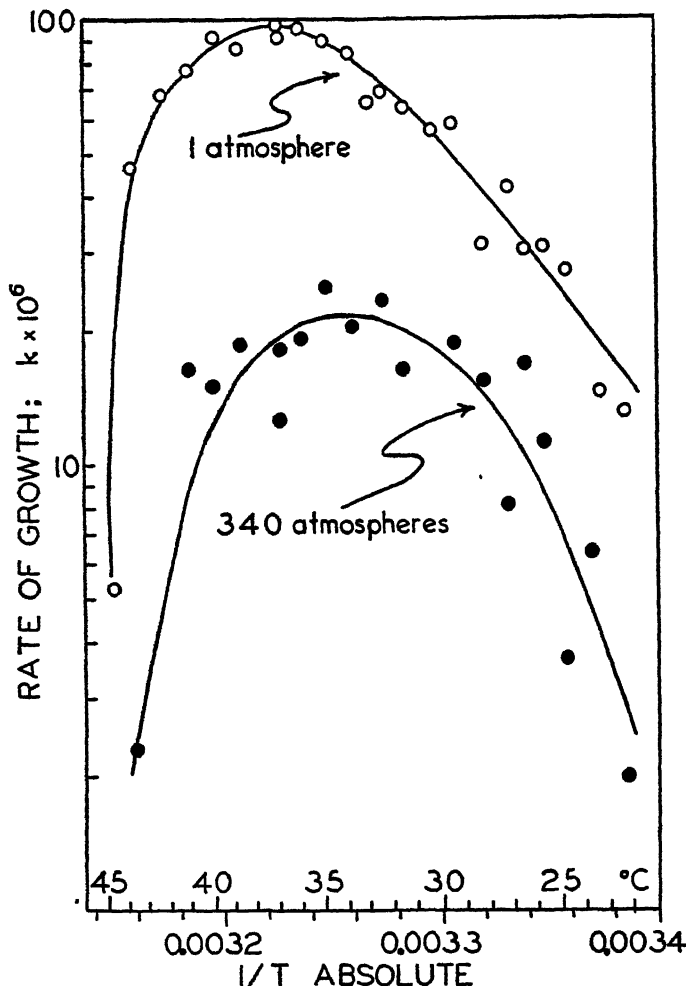


FIGURE 18. Growth (reproductive) rate of *Escherichia coli*, during the logarithmic phase, in relation to temperature, under normal and increased hydrostatic pressure of 5,000 pounds per square inch.³⁵

temperature, at normal pressure, has received considerable attention, the data are not often sufficiently extensive, nor the mechanism of the reactions sufficiently well understood, to justify undertaking an analysis on the basis of the theory described above. According to the results of a recent investigation,³⁷ however, it appears that the rates of oxygen consumption and of methylene blue reduction by *Rhizobium trifolii* are affected by urethane in a manner resembling the action of this drug on bacterial luminescence. Using the same formulations, the quantitative

relation between various concentrations of the drug, and the amount of inhibition at various temperatures, may be accounted for with some accuracy. The influence of pressure in this case has not been studied, but an analysis of its action should assist in elucidating the mechanism of the inhibition.

The evidence that large volume changes of reaction or of activation, respectively, take place in such diverse phenomena as luminescence, extracted enzyme reactions, cell division, protein denaturation, specific precipitation, the action of certain inhibitors, and so forth, would seem to justify more extensive studies from the point of view of hydrostatic pressure as well as temperature. Furthermore, there is considerable reason to believe that the synthesis of complex molecules, biologically specific in structure, involves a templet mechanism, and in order for a molecule to act as a templet it must be in a one- or at most two-dimensional form. This means that globular molecules would have to unfold before functioning as a templet. Such unfolding might be expected to be accompanied by fairly large volume changes. Studies with hydrostatic pressure should yield significant data in this connection also, and the theory worked out with luminescence as an indicator of protein reactivity will perhaps be found useful in various connections.

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THE ANATOMY AND PHYSIOLOGY OF THE LIGHT ORGAN IN FIREFLIES*

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*The histological and cytological work which forms the basis for the original photomicrographs, and for various critical opinions appearing in this paper, was begun in 1938 at the Department of Embryology, Carnegie Institution of Washington, Baltimore, Md., and was continued at the University of Rochester during the period of 1939-1945. The author acknowledges with pleasure the technical assistance of Mrs. Gustav Kuerti, Miss Margaret Ramsay, Miss Olive Pitkin, and Miss Margaret Keister in the preparation of some two thousand microscope slide preparations, part of which was made possible by a grant-in-aid from the Society of the Sigma Xi. Grateful acknowledgment is likewise due to the National Research Council, and to the American Philosophical Society for grants which made possible the author's participation in the 1936 and 1941 expeditions to Jamaica under the auspices of The Johns Hopkins University. Thanks are due also to Miss Inez Demonet and associates for preparing the drawings for this report, and particularly to Dr. Rubert Anderson, Dr. Leigh Chadwick, Dr. E. N. Harvey, Dr. H. Specht, and Dr. Carroll Williams for extensive and helpful suggestions. Appreciation is due to the University of Rochester for generous help with the costs. Finally the author is enormously indebted to Elizabeth Mast Buck for aid with the manuscript.

INTRODUCTION

Since the days of Aristotle and Pliny, and presumably long before recorded scientific observation, the mystery of organic light has aroused the wonder of mankind. Fireflies being by far the most easily accessible luminous organisms, it is understandable that they were probably the earliest and most frequently studied forms. They are also outstanding in their own right, as a subject for scientific observation and experimentation, for perhaps no other animals have luminous organs of such size, brilliance, intricate structure, and physiological complexity. Therefore, it is not surprising that in the past two or three centuries an enormous literature has grown up on various aspects of firefly and glowworm luminescence. It is interesting to see that the problem has proved irresistible not only to specialists on bioluminescence but to a surprising number of men illustrious in other fields, as, for example, Swammerdam, Spallanzani, Davy, Faraday, Humboldt, Darwin, and Pasteur. In this paper, I shall not attempt to cover the literature exhaustively, but rather to review critically the major contributions bearing on the circumscribed problem outlined below.

As our knowledge of living light has increased, and the type of research has shifted from the naturalistic to the quantitative, it has turned out, for one reason or another, that certain aspects of the problem are better investigated on luminous organisms other than the firefly. Thus the crustacean *Cypridina* and luminous bacteria, in particular, have been used in most modern work on the chemistry, kinetics, and enzymology of bioluminescence. For the study of the isolated systems, the very complexity of the firefly light organ is, in a sense, a hindrance. On the other hand, it is well to recall that much of the pioneer work on bioluminescence, even on *in vitro* systems, was done on fireflies. Spallanzani (1796) used the glowworm in one of the earliest demonstrations that luminescence is dependent upon water and oxygen. In the tropical beetle *Pyrophorus*, Dubois (1886) first distinguished the enzyme and substrate of animal luminescence. It was, finally, on fireflies that Coblentz (1912) completed what still stands as the most comprehensive work on the spectroscopy and photometry of light-emission in living organisms. Moreover, fireflies still form the preeminent material for the study of at least two aspects of bioluminescence: the intimate anatomy of photogenic organs, and the physiology of the control of luminescence. This presentation will concentrate on these two aspects.

Since the main task of this paper is the integration of the structural details of the firefly light organ with the various manifestations of light production, it may be helpful briefly to outline the problem from the theoretical side, so that we may have a clear idea of what sort of information will be needed. The most fundamental level of the problem is the chemistry of the luminescent reaction. We need not be concerned with this, except to know the reactants. The next level of the problem concerns the general anatomy of the region in which the luminescent reaction

takes place. Ideally, this would involve knowing where the different reactants are formed or obtained, how they are brought to the scene, and where they are held pending the reaction, or stored if in excess. The final level is the occurrence and control of luminescence. For this, we will need to know how the reactants are brought together at the desired moment, exactly where the reaction occurs, what sorts of luminescences are possible, which of the reactants is made limiting in stopping the reaction, and what physiological mechanisms operate in this control. For the present, we must be content with very incomplete knowledge at each level.

A long series of researches, initiated by Dubois (1885-1919)* and given quantitative expression by Harvey and his associates (Harvey, 1920 and 1940), show that the photogenic reaction involves a minimum of four reactants: water, oxygen, the substrate luciferin (a substance of low molecular weight, probably phenolic), and the enzyme luciferase. For most purposes, it makes little difference whether the oxygen is actually used in the energy-liberating reaction or reactions or in the recovery process.

THE ANATOMY OF THE LIGHT ORGAN

Gross Anatomy. Even externally, the luminous organs of the firefly are so diverse that the only generalizations possible are that they are close to the body surface behind a window of translucent cuticle, and are usually different in male, female, and larva. In size, the organs vary from minute pin-heads to masses occupying the entire ventral surfaces of several abdominal segments. In outline they vary from circular to entirely irregular. In position, they are found from the head to the tip of the abdomen, including the thorax, and on both dorsal and ventral surfaces. A number of representative luminous beetles are shown in FIGURE 1. Most of our common fireflies (*Photinus* and *Photuris*) are of the sort in which the organs occupy sternites 6 and 7 in the male (FIGURE 1, *a*), variable portions of 6, or occasionally 6 and 7, in the female (FIGURE 1, *b* and *e*), and two small spots on the ventral surface of sternite 8 in the larva (FIGURE 1, *c*). This larval position is the same as that in the adults of both sexes of the tropical genus *Diphotus* (FIGURE 1, *d*; Barber, 1941), which fact raises evolutionary questions. Another interesting type is that illustrated by the common European glowworms *Lampyris noctiluca* (FIGURE 1, *r*) and *Lampyrhiza* (*Lampyris*) *splendidula*† (FIGURE 1, *o*),

* In this paper, reference is made only to Dubois' 1886 monograph. Citations of his many other papers can be found in Mangold (1910) and Harvey (1920).

† The taxonomy of fireflies is in a very confused state. Among the points bearing on the subject matter of this paper are the following: (1) Many fireflies, to which the generic name *Lampyris* was applied originally, have since been put in other genera and hence are cited under different names in the older and in the more recent literature (examples: *Lampyrhiza splendidula*; *Luciola italica*, sometimes put in *Phausis*; *Luciola lusitanica*; *Phosphæneus hemipterus*). (2) There exist both *Photinus marginellus* and *Photinus marginellatus*. (3) The famous tropical firefly *Pyrophorus* is an elaterid (click-beetle) and is in a different family from all the other "fireflies" (the beetle family Lampyridae). (4) Because of inadequate descriptions in the literature, Barber was unable to identify, in my collections, most of the lampyrid species previously reported from Jamaica. It is probable, therefore, that some of the species used by Lund (1911) were actually studied by me under new names given by Barber (1941). I have followed custom in using the spellings "*pennsylvanica*" and "*Lampyrhiza*" for the taxonomically correct "*pennsylvanica*" and "*Lampyrhiza*."

on which much work has been done. In these species, the most striking luminosity is seen in the wingless female. Another and very different type of organ distribution is seen in the famous tropical elaterid *Pyrophorus* (FIGURE 1, *m,n*). Both sexes of this beetle have small circular organs on the dorsal posterior corners of the pronotum, which emit a green light when the insect is at rest or walking, and a large rectangular organ in a cleft on the anterior surface of the abdomen, which emits an orange light when the insect is in flight. Some of the most spectacular displays of luminescence are seen in the larvae and larviform females of species in which the adult male is almost or entirely non-luminous. Such a creature is *Phengodes* (FIGURE 1, *s*), which has 11 or 12 segmentally arranged sets of photogenic organs giving a bright green light, and also its South American relative *Phrixothrix* (Harvey, 1944 and 1945), which has, in addition, a red light in its head.

Histological Types of Light Organ Structure. Firefly light organs show an astonishing diversity of structure and can be classified in a number of arbitrary ways. The system used here is an extension of Dahlgren's (1917), based on the arrangement of the tracheae.*

Typically, the photogenic cells are grouped together in one or more compact localized masses with specific tracheal and nervous supplies. However, one exception is found in *Phengodes*, (Type 1), where the light is produced by loose independent giant cells, apparently without tracheae, and similar to or identical with the oenocytes which are widely distributed among and within insects (FIGURE 15; see also Buck, 1940, 1942, 1946a).

The next more complex type of light organ structure is Type 2, found, for example, in *Phrixothrix* (Buck, 1946a), in the lateral "tuberculate" organs of the female of *Lamprorhiza splendidula* (Wielowiejski, 1882; Bongardt, 1903), and the larva of *Phauis Delanouzeei* (Bugnion, 1929). Here (FIGURE 16) the organs are small subspherical compact masses of polyhedral photogenic cells with typically granular cytoplasm, constant in location, and with a specific tracheal supply. This latter consists simply of progressively tapering and more numerous branches, which form a sort of root-system ramifying through the photogenic tissue (FIGURE 8).

The third type of organ is like Type 2, except for the presence of a second layer of cells on the inner surface of the photogenic tissue. This layer is the so-called "reflector layer". Since it will be discussed later, it will suffice at the moment to state that it is composed of cells differing sharply from the photogenic cells in appearance, chemical composition, and staining reaction. This type of organ is illustrated for the larva of *Photuris pennsylvanica* (FIGURE 17) and for an adult *Diphotos montanus* (FIGURE 19) and is also characteristic of the larvae of *Luciola cruciata* and *Pyrocoelia rufa* (Okada, 1935a and b; Hasama, 1942c), *P. analis*

* The profuseness of the tracheal supply external to the photogenic organ has been mentioned many times and is well shown by Gelpel (1915) and Hess (1921).

(Hasama, 1942c), *P. fumosa* (Hasama, 1944b), the larval organs of *Lampyrus noctiluca* (see Wielowiejski and Bongardt), *Lamprohiza splendida* and *Phosphaenus hemipterus* (see Bongardt). The often-studied organs of the adult (female) of *Lampyrus noctiluca* are also apparently of Type 3 (Owsjannikow, 1868; Wielowiejski; Bongardt). In addition, it seems clear, from the work of Heinemann (1886), Dubois (1886 etc.), Geipel (1915), Dahlgren, and others, that both the thoracic and abdominal organs of *Pyrophorus* show the compact but unorganized photogenic and reflector layers characteristic of Type 3, although the much larger size of the organs and much greater thickness of the layers tends to obscure the relationship (FIGURE 18). *Pyrophorus* also agrees with the abovementioned forms in having the simple arborescent tracheal supply seen in Type 2, in which the ultimate tracheal capillaries or tracheoles terminate between the photogenic cells and form the last link in an uninterrupted conduction system leading from the outside (FIGURE 8). The vertical spaces in the photogenic layer shown in Dubois' figure (FIGURE 12) have been doubted by Lund (1911) and Williams (1916) and are probably an exaggerated portrayal of the tendency, noted also by Dahlgren, of the photogenic cells to align in columns. The spaces could be caused artificially by these columns shrinking away from the tracheae running between them (which Dubois failed to see).

All the preceding types of organs differ from those now to be described in that they lack "tracheal end-cells." These cells, which were first described in fireflies by Schultze (1865), are structures which occur at the points where small tracheal twigs narrow rather suddenly and then divide into two or more delicate thread-like tracheoles, or tracheal capillaries, which *appear* to lack the spirally thickened walls which are characteristic of the tracheae proper. These end-cells seem to differ considerably in different material, or according to various workers, as is seen in FIGURES 2, 3, and 11 from Bongardt, Geipel, and Dahlgren, respectively. Nevertheless, there seems to be general agreement that they are uninucleate and have protoplasmic processes or extensions which surround and accompany the tracheoles for varying distances toward or among the photogenic cells, and give the end-cell a stellate appearance, which has led to their being compared with ganglion cells (Kölliker, 1858; Schultze; Eimer, 1872). It also seems clear that the end-cell is the principal site of reduction of inspired osmium tetroxide ("osmic acid") vapor, and, indeed, most of our knowledge of the end-cell was obtained by the use of this reagent (FIGURES 2, 3, and 11). An analysis of the significance of this reduction, and additional details on the internal structure of end-cells, are best deferred. It is worth pointing out, however, that end-cells are widespread in insects, and are even said, by Wielowiejski and Geipel, to occur in non-photogenic tissues of fireflies.

Among organs containing tracheal end-cells, there are a number of different anatomical arrangements which can be classified into convenient, though arbitrary, types. The simplest of these (Type 4) is reported by Dahlgren to occur in "some forms of *Photuris*" (though it does not appear to occur in either *P. pennsylvanica* or *jamaicensis*). As shown in FIGURE 4, the tracheae run ventrally through the reflector layer, as usual. However, when they reach the photogenic tissue, instead of penetrating the latter they divide into several branches which run laterally at the interface between the two layers, terminate in end-cells, and then send tracheoles down into the photogenic tissue, which is only one cell thick.

Another type (Type 5, see FIGURE 5) is found, according to Dahlgren, in the Japanese *Luciola parva*, *L. vitticollis*, and other oriental forms. The tracheal supply arboresces among the photogenic cells as in Types 2 and 3, except that, at certain points where the repeated branching and tapering has reduced the tracheae to quite small tubes, end-cells occur and give off the usual tracheoles. Thus defined, it agrees fairly well with the descriptions of the adult organs of *Pyrocoelia rufa* (Hasama, 1942a) and *Luciola africana* (Geipel), and seems also to include the older descriptions of the adult organs of *Lamprorhiza splendidula* (Schultze, Wielowiejski, Bongardt). The organ of *Luciola cruciata* (Okada, 1935b) is also placed in Type 5 provisionally, although in this organ (FIGURE 9) the larger tracheae appear not to be in direct contact with the photogenic cells, and the arrangement of the end-cells and tracheoles in horizontal section is more regular than expected (FIGURE 9b).

The Type 6 arrangement (FIGURES 7 and 10) is very common, being found in all American members of *Photinus* and *Photuris* which have been studied (*Photinus marginellus*—Townsend, 1904; Dahlgren. *P. pyralis*—Seaman, 1891; McDermott and Crane, 1911. *P. consanguineus*—Williams; *Photuris pennsylvanica*—Seaman; Lund; McDermott and Crane; Williams; Hess, 1922); in some twenty species of Jamaican *Photinus* (Lund; Buck, 1940 and 1942); in *Luciola parvula* (Hasama, 1944b); *Luciola italica* (Tozzetti, 1870; Emery, 1884); and doubtless many others. It is desirable to describe this type in some detail, in order to establish an adequate morphological basis for the future discussions on physiology. As a rule, the Type 6 organs are large, and situated on the ventral surfaces of sternites 6 and 7 in the male and 6 in the female (FIGURE 1, a, b). The reflector layer of the organ is in the inner or dorsal position, while the photogenic layer is ventral (FIGURE 20).

In Type 6, the tracheal trunks which supply the light organ run vertically through the reflector layer as usual, but when they reach the photogenic layer they do not pass directly between the photogenic cells but into specialized cylindrical rods of tissue which go straight through the photogenic layer to its ventral surface (FIGURES 7, 10, 23-26, 31, and 32).

These "cylinders"* contain a number of structural elements in addition to their axial tracheal trunk. First, there are short tracheal "twigs" which branch from the vertical trunk. Second, there is the tracheal epithelium, which, though extremely thin, has fairly large nuclei both along the trunk and the twigs. Third, there are tracheal end-cells, one at the end of each twig, with a nucleus about the size of those of the tracheal epithelium. Fourth are the tracheoles or tracheal capillaries which issue from the end-cells at just about the periphery of the cylinder and run out into the photogenic tissue. Though the existence of a differentiated membrane surrounding the cylinder is not readily apparent in many forms, some preparations suggest such a structure (FIGURES 29 and 38). The integrity of the cylinder tissue as a morphological unit is shown by the behavior of isolated cylinders in maceration preparations from which the photogenic cells have been removed (FIGURES 27, 37, and 40). In addition to these structures, there presumably is some sort of fluid or gelatinous matrix filling the apparent spaces in the cylinder around the end-cells, twigs, etc. Strangely enough, this material does not stain with any technique yet devised, so that in sections of the photogenic layer it is easy to come to think of the clear space around the tracheal trunk as "empty", and of the cylinders as hollow tunnels penetrating the photogenic tissue. If these spaces were indeed gas-filled, our ideas of how the photogenic tissue is supplied with oxygen would need revision.

In horizontal section (*i.e.*, one parallel to the external surface of the photogenic layer), or in surface view, the Type 6 organ shows a beautiful and characteristic "rosette" pattern, which has been remarked by numerous workers (Emery, Townsend, Geipel, Okada, etc.; FIGURES 10, 13, 22, 29, and 36). The vertical tracheal trunks are spaced in regular triangular symmetry, and around each the photogenic cells are arranged like the petals of a flower. In most species, the photogenic cells front upon two contiguous cylinders and have a roughly prismatic or rhombohedral shape.

The Type 6 organ has a number of interesting variations. For example, in many species, the cylinder often flares out like a trumpet or an hourglass at one or both surfaces of the photogenic layer, and there accommodates especially rich brushes of tracheal twigs and end-cells (FIGURES 28, 30, and 39). Such a cylinder in *Photuris jamaicensis* appears in FIGURE 25 in longitudinal section and is well shown in ventral surface view by Okada (1935b). Lund described the photogenic layer of the female of *Photuris pennsylvanica* as a single layer of cells, through which the tracheae run directly without cylinders, and which is penetrated by tracheoles (from end-cells) only from the dorsal and ventral surfaces (FIGURE 14). Such a structure is often seen. In other regions, however, even when only one cell thick, conventional cylinders occur with end-cells

* Called "digitiform acini" by Tozzetti and by Emery.

from which tracheoles run laterally. Possibly, Dahlgren's photurid organ (Type 4) was based on a similar structure in which the end-cells at the ventral surface were overlooked.

The arrangement in *Photinus pallens* is, in one sense, the reverse of that just discussed, in that its cylinder is commonly narrow at dorsal and ventral surfaces but expanded in the interior of the photogenic layer (FIGURE 26). Other species present numerous variations in the relative thickness of photogenic and reflector layers, number of cell layers in each, diameter of cylinders, etc. A few of those occurring in various species of *Photinus* are illustrated in FIGURES 23, 24, and 31. One of the most unusual of these is the organ of *Photinus evanescens montego*, which has an extraordinarily broad and short cylinder, with profusely branched tracheae. A high-power view of this organ shows well the terminations of the numerous tracheal twigs and the limits of the cylinder tissue (FIGURE 31).

One rather puzzling feature of the Type 6 organ concerns the relationship between the processes of the end-cells and the cylinder walls. In *Luciola italica* (Emery), *Photinus consanguineus* (Townsend, Dahlgren), *Luciola parvula* (Hasama, 1944b), and in all of the American and Jamaican species of *Photinus* which I have examined, the end-cells are apparently as figured by Townsend and Dahlgren, and seem to be wholly contained within the cylinder and to give off tracheoles of uniform diameter which penetrate the photogenic tissue alone. In *Photinus marginellatus*, however, which has been studied carefully by Geipel (FIGURES 3 and 6), in *Photuris pennsylvanica*, according to Lund, and in both *P. pennsylvanica* and *P. jamaicensis*, according to my observations, the end-cells appear to have quite thick tapering processes which enclose at least the proximal parts of the tracheoles (FIGURE 32). These processes actually penetrate varying distances into the photogenic tissue, even though the end-cell body itself may lie within the confines of the cylinder or in little bays in the photogenic tissue (FIGURE 14). The arrangement, therefore, resembles in some respects that described in *Lamprohiza* (Type 5) by the earlier workers (FIGURE 2), although there is no doubt that both *Photinus marginellatus* and *Photuris pennsylvanica* have the vertical cylinders characteristic of Type 6. The matter will be considered further under the discussion of end-cells.

The "Reflector" or "Urate" Layer. The reflector layer differs from the photogenic layer in a number of respects. First, the cytoplasm of its cells usually fails to stain with ordinary acid dyes such as eosin, so that in conventional histological preparations the layer is clear, in contrast to the heavily stained photogenic layer. Second, its cells are packed with small, highly refractile particles, probably birefringent and often described as "crystals" or "sphaerocrystals," but also reported by some to have a rounded outline. In sections of fresh tissue, these particles render the

reflector layer opaque by transmitted light, in contrast to the translucent photogenic layer, and white by reflected light, in contrast to the dark photogenic layer. In balsam preparations, on the other hand, the "crystals" are mainly dissolved out by the dilute alcohols and leave the reflector layer nearly hyaline. Other differences, such as solubility in various reagents, have been described (Kölliker, Schultze, Wielowiejski, Bongardt, Townsend; Hasama, 1942a). End-cells have been reported from the reflector layer by Geipel, but according to Lund, Bongardt, and Emery, are very rare or absent.

The two-layered nature of the light organ was first recognized by Kölliker, who described chemical tests purporting to show that the material in the dorsal (reflector) layer is ammonium urate. While there is no reason to doubt this conclusion, and although the work has been repeated a number of times with only minor discrepancies (Owsjannikow, 1868; Tozzetti, 1870; Heinemann, 1872 and 1873; Bongardt; Lund) it has not been checked by modern analytical techniques. In assessing all the work on the chemical composition of the luminous organ or its layers, it must be kept in mind that the two layers have not been separated before testing, so that even microchemical color reactions *in situ* are by no means devoid of the possibility of contamination from the contiguous layer, or indeed from other viscera.

No agreement exists as to the function or functions of the reflector layer. Most workers have accepted, explicitly or implicitly, Kölliker's idea that it serves as a physical reflector to increase the amount of light emitted. No experimental work has been done on this point, however, and in view of the granular type of cytoplasm, I doubt whether the reflecting efficiency of the layer can be very high. In addition, much light would be absorbed in passing back and forth through the photogenic layer. The somewhat similar notion (McDermott and Crane) that the layer may act as a screen to protect the deeper-lying tissues from the light seems likewise to be questionable, because of the lack of evidence or expectation that the light produced in the photogenic cells is injurious.

The presence of urate in the reflector layer has led to a flood of mostly gratuitous speculations on possible metabolic connections between photogenic and reflector layers, even including a contention that the urate granules themselves are the true source of light (Weitlaner, 1909). Although a membrane between photogenic and reflector layers has been described (Tozzetti), the great majority of accounts show the two layers abutting directly (or even with a contact line so irregular that isolated photogenic cells project into indentations in the reflector layer and *vice versa*), so that a direct passage of particulate material between them is at least theoretically possible.

One idea which had considerable vogue provides that the cells of the

photogenic layer, as they get "used up" and filled with the waste products of their activity, are transferred to the reflector layer (Schultze, Wielowiejski,* Dubois; Gerretsen, 1922; denied, however, by Emery and by Lund). Such a process is difficult to visualize for any but the photogenic cells already contiguous to the reflector layer, and would be equivalent simply to shifting the boundary between the layers rather than to actual cell movement. This would require, first, that the photogenic cells closest to the reflector layer, in order to be the first to transform, indulge in the most intense activity, and second, that with time the reflector layer should increase in thickness at the expense of the photogenic layer. Support for either of these points is lacking in most recent investigations, although either proof or disproof would be very hard to obtain, if for no other reason than the great difficulty in assessing and comparing total light emissions. As a matter of fact, "transition" cells† between the two layers have been described (Köl liker; Hasama, 1942a‡; see also FIGURE 23), and Weitlaner (1911) reported an increase in the amount of urate in old as compared with young individuals. On the other hand, Emery found no difference between young and old animals, and Townsend, Lund, and Geipel saw no seasonal change in thickness of either layer. Heinemann (1872) also found no change in four weeks in *Pyrophorus*. Okada (1935a) reported a decrease in thickness of the photogenic layer with age, without change in the thickness of the reflector layer. Hasama, in several species, found no difference in size or distribution of photogenic granules from fireflies long in darkness compared with fireflies after a long period of flashing. Moreover, the presence of transition cells has been denied specifically by several writers (*e.g.*, Lund, Geipel). In view of the considerable variability in layer thickness in different individuals, and particularly in different regions of the same organ (even if care is taken to use only sections exactly perpendicular to the surface), all such work is suspect until confirmed on numbers of specimens adequate to demonstrate statistically significant differences.

A somewhat similar idea is that the urate granules represent waste products of the oxidation of the photogenic granules in the light cells, which have been transferred by an undescribed process to the reflector layer and there stored (Köl liker, Tozzetti, Lund). Lund has championed this view strongly. His claim that the "waste product" deposited in the dorsal layer is "allied to or identical with some of the split products of nucleic acid" is interesting in view of the recent demonstration that firefly light organs contain a relatively high proportion of flavin-adenine

* Wielowiejski, in a later paper, came to the opposite conclusion in regard to American fireflies.

† Not to be confused with the term, transition cell, used by Holmgren (1895) and Townsend as synonymous for end-cell.

‡ Bongardt saw cells intermediate in character, but not at the boundary between the layers.

dinucleotide (Ball and Ramsdell, 1944).* However, not only is there no decisive evidence of an actual transfer of material between layers, but, as Okada (1935a) has pointed out, there are difficulties in explaining how a waste material, which (according to Lund) crystallizes in the photogenic cells, comes to be redissolved, transferred (there is no blood circulation directly between the two layers), and recrystallized in the cells of the urate layer. Furthermore, Lund's "striking and conclusive" proof of a "direct relation and actual tracing of products of decomposition resulting from photogenesis from their place of origin in the photogenic cells into the dorsal layer cells" consists of no more than the following observation: When a cross-section of an entire photogenic organ is viewed by reflected light, the dorsal (reflector) layer was seen to be packed with a dense white mass (crystalline deposit), and in the ventral layer similar ("same") material was seen in the most peripheral cytoplasm and sometimes around the nuclei of the photogenic cells. Small amounts of this material were also observed by Lund in other viscera, and other investigators have reported that "urate" occurs in the fat body in considerable concentration. Lund also claimed that he found "different amounts of the accumulated products of katabolism in different species and different specimens of the same species," and that "the degree of filling of the dorsal layer cells also corresponds to the amounts of the deposit upon and in the photogenic cells." These claims are in direct opposition to the observations of several workers, particularly Emery, and Geipel. By implication, Lund regarded these differences as progressive stages in a process starting with no crystalline waste in the photogenic cells at the beginning of adult life, and culminating in a heavy deposit in aged specimens.

Lund's theory implies that at any moment the photogenic cells of a given firefly would present a cytoplasmic appearance directly related to the total amount of light which had been produced. Since such an assumption is prerequisite for all ideas of photogenic-urate transformation, it is germane to point out, first, that with a number of techniques a considerable cytoplasmic variation is apparent not only between the photogenic cells of the same individual but between contiguous cells, and second, that the finding of differences does not necessarily justify arranging them artificially into an irreversible temporal sequence. It seems more reasonable to assume that the photogenic cells go through individual, and probably asynchronous, cycles of activity which are repeated a number of times during the functional life of the photogenic layer. Such a view

* It is also interesting in view of McElroy & Ballentine's (1944) claim that phosphate is liberated during the *in vitro* luminescent reaction of *Cypridina*. McDermott (1915) attempted to test Lund's hypothesis by comparing the soluble phosphorus and nitrogen content of a solution of dried and powdered firefly organ which was allowed to luminesce in the presence of peroxide, with that of one prevented from luminescing. He found slightly less of both phosphorus and nitrogen in the solution which had luminesced, and concluded that Lund's theory was unsound. It is not clear, however, that a change in phosphorus and nitrogen should have been anticipated in McDermott's experiment.

would agree with what occurs in many gland cells. At present, we can only speculate on what this "activity" is. However, the schemes of enzymatic oxidation and resynthesis of luciferin which have been developed in recent years furnish a reasonable pattern (see summary in Johnson *et al.*, 1945). It is true that the cytological differences between cells of the same organ could be explained by assuming that all the cells are in a stable equilibrium of oxidation (photogeny) and synthesis, but that the point of equilibrium (intensity of activity) differs in different individual cells and is reflected in their appearance. Critical information bearing on these various possibilities would be of great importance in questions of chemistry and physiology of the photogenic process and the mechanism of control, but none exists aside from scattered observations of spotty or localized glowing of the photogenic organ. These, however, indicate the possibility of differences in the responses of different cells.

The fact that organs of Types 1 and 2 operate without a reflector layer could be construed as indicating that it cannot have any essential connection with photogeny, although Wielowiejski argued that, since these organs are characteristically very small, their wastes could diffuse away directly.

Recent biochemical schemes (*e.g.*, McElroy and Ballentine, 1944), provide for the resynthesis of at least part of the oxidized luciferin. If this occurs, and if the necessary energy is supplied by reactions having gaseous or easily diffusible end-products, there need be no accumulation of organic waste products in the photogenic cell and no necessity for providing elaborate mechanisms for their removal. There is also the possibility that reactions which are not reversible *in vitro* (*e.g.*, the luminescent oxidation of *Cypridina* luciferin) are reversible *in vivo*, making possible an even greater conservation of material by cyclic re-utilization.

On the whole, therefore, there has been no clear demonstration of what the function or functions of the reflector layer are, although the large bulk of the tissue, its close association with the photogenic layer, and its regular presence in many fireflies suggest that it must be of utility to the organism.

The Tracheal End-Cell. The end-cell has been the subject of much detailed study, since its strategic position at the beginning of the tracheoles at once suggests that it may be concerned in controlling the oxygen which, presumably, passes through the tracheoles into the photogenic cells. The end-cell is by no means a typical cell. Ordinary techniques often fail to reveal much more than its nucleus. The cytoplasm is best demonstrated by subjecting the intact animal to osmic acid vapor. This apparently diffuses unaffected through most of the tracheal system, but escapes into the cytoplasm in the region of the end-cell, where it is reduced to the black suboxide or metallic form. Progressively heavier doses delineate

progressively more of the cytoplasm and processes of the end-cell, and sometimes the tracheoles. The number of processes present usually corresponds to the number of tracheoles given off, where the two are distinguishable. It varies from two to seven in different species, though it is usually rather constant intraspecifically.

It is sometimes not clear how the tracheoles originate within the end-cell. Geipel shows the tracheal twig terminating blindly within the end-cell, without any tracheoles being present (FIGURE 3). However, the most popular idea involves a continuous tubular connection, within the end-cell, between the narrowed tip of the tracheal twig (or a common tracheole issuing from this tip), and the tracheoles (see FIGURES 2, 11, and 13; also the drawings of Wielowiejski). According to Bongardt, and Dahlgren, there is a darkly staining annular swelling ("rounded body" or "ampulla") around the common tracheole (FIGURE 11, "S"), for which sphincter properties have been postulated (Dahlgren; Creighton, 1926; Snell, 1932; Alexander, 1943). Little differentiation has been observed in the cytoplasm of the end-cell, except by Dahlgren, who described "contractile radial fibers" (FIGURE 11). This observation is considered in detail in the last section of the present paper.

Lund seems to have regarded the permeability to osmic vapor, and the ability to bring about its reduction, as specific and exclusive properties of the tracheal end-cell and the tracheolar wall or membrane. On the basis of the temperature lability of the osmic reduction effect, he even ascribed it to the presence of a specific "reductase", which he assumed also to perform an analogous function in transferring oxygen in luminescence.* Wielowiejski had a somewhat similar idea. However, it has long been known that, with heavy doses, the area of reduced osmium spreads beyond the end-cell into the cytoplasm of the contiguous photogenic cells. Moreover, it was shown by Wielowiejski that, in Type 3 organs (which lack end-cells, and in which the tracheoles do not all originate at one point), the tracheoles are nevertheless permeable to osmic acid vapor and the surrounding photogenic cytoplasm is able to accomplish its reduction.† This was also shown clearly in *Pyrophorus* by Geipel.

* Remy (1925) found (not in fireflies) that the tracheal wall recolorizes indigo-white and, hence, has an "oxidative power." It is not clear, however, why this effect is not due merely to the proximity of gaseous oxygen. Aside from luciferase, no enzymes have been separated from photogenic tissue. Among other enzymes which have been postulated is Gerretsen's "photogenase", which presides over the formation of luciferin. Some suggestion of catalase activity is contained in Burge's (1916) claim that fresh firefly tissue liberated more oxygen from hydrogen peroxide than did an equal weight of bee tissue, and that the "luminous part" of a firefly liberated more than the non-luminous. The work, however, was poorly controlled.

† Wielowiejski distinguished clearly and categorically between Type 3 organs with their tree-like tracheal branching (such as those of *Lampyrus* or the minute "tuberculate" organ of the female of *Lamprohiza splendidula*) and the Type 5 organs with end-cells of *Lamprohiza splendidula*. He did point out, however, that sometimes in Type 3 organs the point of origin of tracheoles, especially if more than one tracheole is involved, may simulate an end-cell due to the reduction of osmium there. This is particularly true since the thin tracheal epithelium often fans out at such loci. Geipel apparently observed similar structures in the reflector layer of *Photinus marginellatus*, which he interpreted as a different type of end-cell. Bongardt, working with the same materials as Wielowiejski, apparently duplicated the latter's observations very closely, but his presentation and discussion are so incredibly incoherent and contradictory (culminating on his page 25) that I found it impossible to make out whether he even believed in the existence of end-cells at all.

One of the least clear features of the end-cell is its external surface. This subject, though rather esoteric, is considered here in some detail, because it may help to clarify a large mass of confused literature and because it bears on certain properties of the end-cell and tracheoles which are of physiological interest. In macerated preparations (FIGURES 27, 37, and 40), the end-cells maintain their integrities as separate masses of protoplasm. However, their apparent limits seem often to depend on the degree of osmic impregnation (*e.g.*, Schultze, Townsend, Lund) and often no sharp membrane can be made out in either fixed or fresh preparations. This fact is illustrated in Townsend's and Dahlgren's figures, and I have observed it in most of the species of *Photinus* which I have studied (FIGURES 31, 37, 39, and 40). Emery was even led to consider all the contents of the cylinder as a sort of syncytium, and both Emery and Townsend regarded the end-cells as artifacts. It is clear from their figures, however, that they were dealing with the same structures which we now call end-cells. On the other hand, many investigators figure the end-cells as with a definite boundary, although they are vague about its nature (*e.g.*, FIGURES 2 and 3). Part of the confusion results from the fact that the tracheal end-cell is believed to be a derivative of the thin tracheal epithelium which covers all the tracheae, including the lateral twigs in the cylinder (Lund; Williams; Hess, 1922). Wielowiejski and Emery, for example, conceive of this epithelium as stretched over the flat fan-like furcation point of the tracheoles, like the web of a duck's foot, thus forming the "membrane" of the end-cell. This membrane, in turn, is produced as an "end-cell process", and invests each tracheole individually throughout its course (or, according to Bongardt and Williams, part of its course). Over most of this distance, the epithelium is indistinguishable from the tracheolar wall proper, because of its extreme thinness. On the other hand, in regions where the two can be separated visually, they can be differentiated also by the use of strong alkali, which dissolves the former, leaving the chitinous (*sic*) tracheole.

Other descriptions of end-cells differ in several respects from that of Wielowiejski. According to Bongardt, the end-cell processes accompany the tracheoles for long distances in the photogenic tissue, branch and anastomose richly on the surfaces of the cells, but eventually terminate and leave the individual unbranched tracheoles to run alone farther into the tissue. Geipel, on the other hand, seems to find no typical tracheoles at all in *Photinus marginellatus*, but only long, hollow, tapering end-cell processes, like the roots of a molar tooth, which embrace the photogenic cells (see also FIGURE 10).^{*} Likewise, most of the species studied by Hasama have tapering end-cell processes. Here, however, a tracheole runs in each process up to, but not beyond, its tip. My observations on *Photuris*, which has end-cells apparently very similar to those of *Photinus*

^{*} The tracheoles shown within the end-cell and processes of Dahlgren's reproduction (his FIGURE 19) of Geipel's figure (here reproduced as FIGURE 3) are not in Geipel's original.

marginellatus, lead me to believe that Geipel overlooked all, and Hasama part, of the tracheoles. In *Photuris*, these extraordinarily delicate tubules can be seen running from the tracheal twig into each end-cell process, and then issuing from the distal end of each process and proceeding farther into the photogenic tissue. This is visible in preparations made by several standard methods, and thus is unrelated to the vagaries of osmic acid penetration.

In summary, it seems justifiable to conclude that all the types of end-cells discussed are built on the same fundamental plan of multiple tracheolar branching from a tracheal twig.

There seem to be two main types: the *Photinus* type (with some exceptions), in which the end-cell is strictly limited to the cylinder, has an indistinct "cell membrane", and gives off usually two tracheoles of uniform diameter (FIGURES 11, 22, 26, 31, and 38); and the *Photuris-Lamprorhiza-Photinus marginellatus* type, in which the end-cell projects into the photogenic tissue, has a definite "cell-membrane", and gives off usually four to seven apparently tapering processes which accompany the tracheoles for varying distances into the photogenic tissue (FIGURES 6, 10, 14, and 32). There are numerous additional minor discrepancies between the various accounts of end-cells. Some of these are doubtless *bona fide* differences in material. However, there is probably considerable justification for attributing much of the contradiction to inferior optics, since the homogeneous immersion lens was not in general use until about 1885. This is the more reasonable in that the details in question are near the limits of microscopic resolution.

The Photogenic Cytoplasm. The strikingly granular contents of the photogenic cells have attracted the attention of numerous workers. The granules are minute, usually spherical and densely packed, and take acid or plasma dyes. Dahlgren claims that tissues fixed in boiling fixatives show that the granules are spherical in the male, rod-shaped in the female (FIGURE 11). Hasama (1942a) could not confirm the distinction in *Pyrocoelia rufa*, but says that Okada found about half the females in *P. consanguineus* differing from the males in the manner claimed by Dahlgren. In *Photinus pallens*, I found that granule shape is more or less constant in a given individual and may differ in different individuals, but is not characteristic of either sex.

Dubois regarded the photogenic granules ("vacuolides") as self-perpetuating entities comparable to mitochondria, and a similar concept is implied in Dahlgren and Kepner's term "photochondria". Vonwiller (1921) claimed that the granules stained like mitochondria, but Takagi (1934) showed that mitochondria could be demonstrated independently of the granules in *Luciola cruciata*. Moreover, Dubois was unable to culture granules isolated from several organisms.

Kuhnt (1907) suggested, by analogy with leguminous root-nodules,

that the firefly light organ contains symbiotic bacteria. Pierantoni (1914), influenced by his studies on the symbiotic "mycetome" organs of Hemiptera, claimed to have cultured two kinds of bacteria from both the photogenic organ and the egg of *Lampyrus*. These "bacteria" were described as distinct from the photogenic granules. Their figured appearance strongly suggests that they are mitochondria. Pierantoni did not make single-cell isolation cultures, and his evidence for the transmission of the "bacteria" by way of the egg is unconvincing. Furthermore, his cultures were not luminous. This is not necessarily a decisive objection in view of the known dependence of bacterial metabolism on culture conditions. Also, similar results have been obtained in various organisms, particularly fish, where the evidence for bacterial symbioticism is much more sound than it is in fireflies (see Harvey, 1940, pages 30 to 36). Vogel (1922) and Hasama (1942a) were unsuccessful in culturing anything from the light organs of fireflies, although Hasama's failure may have been influenced by his choice of 37° C. as an incubation temperature. Buchner originally (1914) supported Pierantoni's thesis, though he never, himself, worked on Lampyrids. In the second edition of Buchner's book (1930), however, the matter is left undecided.

Harvey and Hall (1929) demonstrated that the development and functioning of the adult light organ in *Photuris pennsylvanica* is unaffected by ablation of the larval organs. They concluded, therefore, that bacteria are not concerned with luminescence. They acknowledged, however, the possibility that bacteria in a non-luminous phase might have existed in other parts of the body and have contributed to the formation of the adult organ.

On the whole, although the photogenic granules and other cytoplasmic inclusions often resemble bacteria in form, size and staining, and although some organisms apparently do have organs (even luminous organs) which contain symbiotic bacteria, the evidence in regard to fireflies is so weak that a definite conclusion would be wholly gratuitous.

A number of workers (e.g., Williams) have described the staining reactions of the photogenic granules with a variety of dyes. Since, however, most such reactions are highly unspecific in a chemical sense, the most that can be concluded is that the granules probably contain protein. The granules have been identified as "the" photogenic material (Dubois; Dahlgren and Kepner, 1908; Lund, McDermott, Dahlgren, Williams, etc.), or even specifically as "luciferase", "photogenin" or "luciferin", with very tenuous justification except that granules of some sort seem to be associated with light-production in all known animals. It is outside the scope of this paper to go into the chemistry of the photogenic layer in detail, but it is obviously relevant to mention that analyses of whole organs by Dubois, Harvey, Gerretsen (1922), McDermott, and others, have shown that luciferase and luciferin are present (or, at least, that

two extracts can be prepared which luminesce when mixed). These are universally assumed to be localized in the photogenic layer. Considering the certainty that the photogenic layer contains a number of compounds of varying complexity, and in view of the probability of contamination already mentioned, it is not surprising that earlier analyses of the material of the "photogenic layer" yielded identifications as diverse as "albuminous" (Kölliker), "lecithin-like" (Lund), and "phosphatide with an aliphatic radical" (McDermott, 1911b).

Another interesting feature in the cytoplasm of Type 6 photogenic layers is the striking "differentiated zone" which surrounds the cylinders, (figures by Lund; Hess, 1922; Dahlgren; Williams; and Okada, 1935b). This zone is formed by a part of the peripheral cytoplasm of the photogenic cells in which the so-called photogenic granules are lacking, and only an extremely fine-grained and compact-looking cytoplasm can be seen (FIGURES 10, 11, 22, 26, and 31). The zone is of varying thickness in different species and in some has apparently not been recognized. That it differs chemically or physically from the rest of the cytoplasm, is indicated by its different response to a number of dyes. It is traversed, of course, by the tracheoles or by the end-cell processes as they pass from the cylinder into the luminescent tissue. Thus it is, by inference, the region where photogenic material might first come in contact with oxygen. According to most workers, this differentiated region is not found between different photogenic cells but only between the cells and the cylinder, or along the dorsal and ventral surfaces of the photogenic layer (FIGURE 26). In some of my preparations, however, it apparently also forms a very thin layer along the internal faces of the photogenic cells. The zone thus envelops the photogenic cells completely and "insulates" their interior cytoplasm. Possibly with this thought in mind, Dahlgren made the stimulating but unsupported proposal that this zone is impermeable to oxygen and serves as a protection against the "entrance of any oxygen that might come into the cells except through the tracheal capillaries." Aside from the difficulty of visualizing the mechanism of this impermeability, many firefly photogenic organs are apparently not equipped with such a protective layer and yet do not show uncontrolled luminescence. There may be some merit in the general concept, however, since Wigglesworth (1930), and others have shown that the walls of even large tracheae are permeable to oxygen. This suggests, in view of the low partial pressures of oxygen necessary to support luminescence (see page 442), that the quantity dissolved in the general body fluids might be sufficient to support luminescence, if there were no insulation. Also, as suggested on page 424, the differentiated layer might possibly have some bearing on observed differences in types of luminous emission (see also page 449).

The final peculiarity of the photogenic cytoplasm here considered is the unobtrusiveness of its limiting membrane. To be sure, the presence of

sharp cell boundaries is described, or implied, by numerous workers (e.g., FIGURES 6, 9, and 10; see also Hasama, 1944b), but in other instances tribute is paid to the fact that often no membrane of any sort has been observed (FIGURES 7, 11, 14, and 23). Lund, in fact, frankly regards the photogenic cytoplasm as a syncytium. This may be so in some species, but I hope to show later that in *Photuris pennsylvanica*, to which Lund referred particularly, cell membranes can be demonstrated. One reason why the membranes are difficult to find in Type 6 organs, is that they are concealed or obscured by the tracheoles. For example, in a horizontal section of the photogenic layer, the "lines" delineating the rosette pattern ordinarily appear single, and it is virtually impossible to decide, even with progressive focusing, whether one is looking at a tracheole or at a cell membrane in edge view (FIGURE 22). Even a double line would not be conclusive, since "the" membrane separating two photogenic cells is presumably double. Likewise, in a cross-section of the organ, some membranes are in face or quartered view, and hence invisible. Usually, it is only when a section is chosen so that an edge view of a membrane and a cross-sectional view of tracheoles are seen simultaneously, that one can distinguish the two with certainty. Figures of such views have been published by Bongardt, Townsend (and Geipel, for the end-cell processes), and examples from *Photuris pennsylvanica* and *Photinus pallens* are presented in FIGURES 35 and 36. In horizontal sections of the organ, cross-sections of tracheoles can usually only be seen close to the ventral (outer) surface, since it is only here that many of the tracheoles run vertically (FIGURE 36).

The Tracheoles. The origin of the tracheoles and their general arrangement should be clear from the discussions and figures already presented. Measurements of the dimensions of tracheoles (or, indeed, of any part of the luminous organ) are rare. The only figures for tracheolar diameter are Wielowiejski's and Lund's 1.3 and 1.1 microns, respectively, and for length, Townsend's value of 20 to 60 microns (estimated from her figures on the distances between cylinders in *Photinus marginellus*). The quoted dimensions are of the same order of magnitude as the averages obtained by the writer, but there are wide differences between different species, and the diameter measurements are probably subject to large errors as well as to variations caused by different techniques of preparation. In *Photuris*, the tracheoles issuing from the ends of the end-cell processes appear to be much less than a micron in diameter. In organs of Types 2, 3, and 5, where anastomosis probably does not occur, the tracheoles are much longer and by no means uniform in diameter at their origins. Moreover, they taper, so that diameters approximating or exceeding the resolving power of the microscope may be reached near their distal ends.

One hotly debated morphological point, which has physiological impli-

cations also, is whether the tracheoles actually penetrate the cytoplasm of the photogenic cells or are exclusively extracellular in their courses. Heinemann (1872) reported that the tracheoles impale the photogenic cells in *Pyrophorus*, though Robin and Laboulbène (1873), and Geipel described them as applied to the cell faces. Lund strongly espoused intracellular penetration in the several lampyrids he studied. However, the majority of workers on lampyrids have reported that the tracheoles (or end-cell processes) run only between or on the outside surfaces of the photogenic cells (Wielowiejski; Emery; Watasé, 1895; Bongardt; Townsend; Geipel; Dahlgren; Williams; Okada, 1935b; and Hasama). Both intracellular and extracellular tracheoles have been reported in other kinds of insects (Wigglesworth, 1930 and 1939). Lund's stand was primarily based on finding tracheoles close to nuclei in the same focal plane, mainly in *Photuris pennsylvanica*. I think this observation was a misinterpretation, due to the fact that in this species, the limits of the cells are poorly delineated and the cells are often irregular in shape, overlapping, and scarcely wider than the nucleus (FIGURE 35). At any rate, I have found no case of intracellular penetration of tracheoles in any of some twenty Jamaican and American species of *Photinus* and *Photuris*. Perhaps the most convincing evidence comes from the type of preparation already referred to, in which cell membranes and cross-sections of tracheoles both appear (FIGURES 35 and 36). Another type of evidence is seen in surface views of freshly extirpated light organs which have been dried sufficiently for air to enter the tracheoles. Here, the tracheoles follow a course similar to that figured by Townsend (FIGURE 13), which corresponds exactly to the intercellular interfaces seen typically in the rosette pattern of horizontal sections (FIGURES 29 and 38).

Another disputed subject is whether the tracheoles end free or anastomose with other tracheoles. This has physiological implications, since anastomosis might permit gas flow through the tracheoles, whereas transport to free endings would almost certainly be by diffusion. Anastomoses have not been reported in organs of Types 2 and 3. In *Lampyris* and *Lamprohiza* the existence of "loops" was claimed by Kölliker, and denied by Schultze, but since tracheoles were unknown at that time, it is not clear what was meant. However, Wielowiejski found anastomoses only occasionally, and Bongardt states that they do not occur.* Five of the apparently Type 5 species discussed in Hasama's papers are likewise of the *Lampyris* type, since the end-cell processes (which are said to wholly contain the tracheoles) do not anastomose (*Pyrocoelia rufa* and *P. analis*; *Luciola lateralis*, *L. cruciata* and *L. gorhami*). In most Type 6 organs, profuse tracheolar anastomoses between contiguous cylinders are described (McDermott and Crane, Lund, Townsend, Williams, and Buck, 1942). Emery saw no anastomoses in *Luciola italica*, though his FIGURE 7

* However, Bongardt described the tracheoles as running in the end-cell processes, and the latter as branching and anastomosing richly!

closely resembles most Type 6 organs (e.g., FIGURES 13, 29, and 39). Tozzetti apparently saw some anastomosis *within* the cylinder in *L. italica*, although his descriptions and figures are not entirely clear. Okada (1935b), however, shows the end-cell processes ending free (FIGURE 10). On the whole, there appears to be a systematic difference between Types 5 and 6 in regard to anastomosis of tracheoles. This should be kept in mind during the discussion of flashing behavior in the two types.

[*Note Added in Proof.* Dr. A. Glenn Richards recently made a number of electron micrographs of tracheoles from fresh light organs from males of *Photinus pyralis* which I supplied. The prints (3800 X) show isolated tracheoles of very uniform diameter (about $0.25\ \mu$) and up to $25\ \mu$ long. In accordance with Richards's findings in many other insects, the tracheoles show clear spiral thickenings throughout, and appear to end blindly, without anastomosis. I have some doubts as to whether the tracheoles are actually those originating from end-cells in cylinders, because in several instances they appear to arise in groups of three, whereas in histological preparations the number is invariably two. Moreover, the advantages devolving from the increased resolution of electron optics are somewhat offset by the necessarily drastic method of preparation (maceration in water or weak alkali, teasing, drying *in vacuo*, and exposure to electron beams). Nevertheless, Richards's pictures caution against any dogmatic insistence on the existence of tracheolar anastomosis.]

Several writers have quoted Schultz as having shown the tubular nature of the tracheoles by gold chloride impregnation, forgetting that he never saw the true tracheoles. Wielowiejski, however, demonstrated the lumen by infiltration with dyed soap. It is now generally agreed both that they are hollow, and that the tracheolar wall is exceedingly thin. Both facts are illustrated in those figures of Bongardt and of Townsend which show cross-sections of tracheoles, and in FIGURE 36.

Though all agree that the tracheoles are hollow, there is less unanimity on what they contain, at least in life. Schultz, Tozzetti, Emery, and Lund claimed that in freshly dissected photogenic tissue the "tracheoles" (in some cases the finer tracheae must be meant) are nearly or entirely invisible, because they contain liquid. Tozzetti suggested that the tracheoles might carry part of the blood circulation. Wielowiejski and Townsend found that glycerin enters the air-filled tracheoles of a dried organ from the inside out (i.e., distal-proximal direction), from which they concluded—irrelevantly, it seems to me—that the tracheoles are air-filled in life. No observations have been made on live fireflies, nor are any to be expected, in view of the thickness and delicacy of the photogenic tissue. On the other hand, in insects such as fleas, and mealworm and mosquito larvae, which, admittedly, have different anatomies, the situation in life is apparently quite variable and changes under various conditions (Wigglesworth, 1939). It is clear that, in some

instances, the tracheoles are normally air-filled down to diameters of 0.5 micron and, since this is the order of magnitude of firefly tracheoles, there seems to be no reason why they might not be air-filled. As a matter of fact, unless the tracheoles were gas-filled throughout, intracellular penetration of tracheoles would confer no advantage, since the distribution of oxygen in the photogenic cytoplasm (if such is the purpose of the tracheoles) must ultimately occur by aqueous diffusion, which could only be hampered by a tracheolar wall. This matter will be further discussed in the section on physiology.

The Nerves. The least-known major anatomical feature of the photogenic organ is its nerve supply. In spite of contrary statements in the literature, I have not found the ganglia supplying the photogenic segments to be disproportionately large in comparison with those of similar non-luminous insects. K  lliker and Schultze were the first of several to report having seen nerves in the photogenic organ. With rare exceptions, however, these structures were demonstrated by reagents and techniques which are not recognized today as having diagnostic value in the identification of nervous tissue. Therefore, since even the standard neurological techniques are too often unpredictable, the possibility of artifact deserves serious consideration. This is the more pertinent in view of the great difficulty reported in demonstrating the "nerves" and especially in distinguishing their fine branches from tracheoles. Also, some very atypical structures have been described, as, *e.g.*, the "knobbed" and "multinucleate" fibers of Wielowiejski and Bongardt.* Wielowiejski devoted several pages to a description of connective tissue fibers (in non-photogenic tissue) which previous workers had misidentified as nerves, indicating that the techniques then in use were by no means reliable.

However, taking the reports at face value, the nerves generally follow the tracheal system rather closely (though Wielowiejski denies this) and are distributed in roughly the same fashion. There is much disagreement as to the ultimate terminations of the nerves, which are, of course, the regions of greatest interest. Tozzetti and Emery found no connection between nerves and any sort of cells in *Luciola*. K  lliker and Schultze could not trace the finer nerves to their ends in *Lamprohiza*, though Schultze thought it likely that they innervated the photogenic cells. Wielowiejski found direct connections between nerves and the surfaces of photogenic cells in *Lamprohiza*, as did Owsjannikow (1868) in *Lampyrus*. Owsjannikow even described the nerve as penetrating to the nucleus, though Wielowiejski decried this idea. Geipel and Hasama (1942a) reported that in *Photinus marginellatus* and *Pyrocoelia rufa*, respectively, the finest nerves connect directly with the end-cells (to the

* Lindemann (1868) described "nerves" running to the "light-balls" in *Lampyrus*, but his descriptions and conclusions are so bizarre that I can only conclude that he had mistaken some other tissue for the photogenic organ.

nucleus, according to Geipel), and in *Lamprorhiza* and *Lampyris*, Bongardt found them connected with the exterior of both end-cells and photogenic cells.

There is, therefore, no general agreement on the details of the nervous supply of the photogenic organ. In my opinion, a really convincing answer to this problem will require a full investigation which is devoted to this one point and makes use of modern neurological techniques.

A Possible Ultra-tracheolar Network. In preparations made by silver nitrate impregnation, the tracheoles are heavily outlined in black by precipitated silver. In cross-section, they appear as little black circles strung on dotted black lines which are formed by a light deposit of silver on the photogenic cell membranes (FIGURE 36). Wherever the plane between two tracheoles (which means the plane of a cell membrane) is horizontal or nearly so (that is, parallel with the stage of the microscope), the tracheoles are seen to be knit together by a close-meshed network (FIGURES 33, and 34). This remarkable structure is only visible here and there at any one focus in a given field, in agreement with the rarity of instances where a flat region of cell membrane happens to lie parallel with the surface of the section, but it is often possible to trace the network over a relatively large area by careful focusing. The individual strands of the network are far too slender (of the order of 0.1-0.2 microns) to make it possible to say whether or not they are tubular. I have found them in *Photuris pennsylvanica* and *P. jamaicensis*, and in *Photinus pyralis* and *P. pallens*.

This network could be interpreted as a mesh of extraordinarily fine tubules binding the tracheoles together, in a manner analogous to the capillary networks between arterioles and venules. This might make a more satisfying picture, physiologically, than tracheoles alone, because such a network might permit quicker and more uniform distribution of oxygen to the photogenic cell, and thus reduce the partial pressure of oxygen necessary to support luminescence. However, in spite of the sharpness, relative orderliness, and wide distribution of this structure, I am not insisting on the above interpretation for the present, because metallic impregnation is such a notoriously capricious technique that we must reckon with the possibility of an artifact. Furthermore, in maceration preparations, and in those prepared with caustic, where the tracheoles are relatively free from other tissue, they do not behave as if held together by a network, nor do they show a rough outline such as might be left if the network had been torn off.

On the other hand, "ultra-tracheolar" offshoots of tracheoles have been figured from other insects, though not in a network (Wigglesworth, 1939, FIGURE 164).^{*} The reason that the network appears only with the

^{*}The networks described by Wistinghausen (1895) and Holmgren are larger than those discussed here by a factor of ten. Other supposed tracheolar networks have been reported but have been shown to be present also in vertebrate tissue (see Wigglesworth, 1931).

silver nitrate technique is undoubtedly that the "tubules" are so delicate that they would be invisible without a completely opaque coating. This probably explains, also, why they have not been reported previously, since silver nitrate has been used on firefly organs only very rarely (Owsjanikow, 1868, Tozzetti; Geipel).

PHYSIOLOGICAL ASPECTS OF LUMINESCENCE

Introduction. It was formerly customary for writers on fireflies to include a section or appendix on "physiology." Unfortunately, far too many of these were concerned only with a perfunctory and uncritical exposure of fireflies to various common laboratory reagents. Some of the early work, nevertheless, led to fundamental conclusions in spite of the understandable crudeness of the experiments. In considering modern physiological theories, we shall make extensive use of two of these general findings: the respective influences of the nervous system and of oxygen on luminescence.

The remaining "physiological" literature falls into three classes. The first deals with the actual nature of bioluminescence. These old arguments and experiments over whether fireflies light by means of phosphorus, crystallization, phosphorescence, etc., are interesting historically, but of no relevance to the present discussion since it has been generally agreed for over fifty years that animal light involves an enzymatic oxidation of an organic substrate. The second class of literature concerns the question of whether or not the light is a vital phenomenon, and again this can be dismissed, for no one since Pflüger* (1875) and Bellesme (1880) has doubted that the actual luminescent reaction can occur in the absence of living protoplasm, or even *in vitro*. Finally, there is a huge mass of heterogeneous and unsystematized work which concerns almost every aspect of bioluminescence. Included in this category, for example, are the work on the effects of temperature, the papers on the spectral character of the light (for review, see Buck, 1941, and Grinfeld, 1944), and particularly the reports on the effects of literally hundreds of gases, vapors, acids, bases, salts, poisons, drugs, solvents, excretions, enzymes, etc., on intact fireflies and on isolated organs. All this work suffers from the fundamental ambiguity that it is uncertain whether the agent is acting directly on the actual luminescent reaction or upon a biological mechanism controlling it, as, for example, the nervous

* The facts usually cited as evidence that light-production can be independent of life are the following: (1) Fireflies may still be glowing several days after their apparent deaths. (2) Fireflies which have been quickly dried and kept in absence of air, may glow when moistened, even after several years (*e.g.*, McDermott, 1916). Pflüger (p. 287) countered these two arguments with allusions, respectively, to the sustained irritability of extirpated (surviving) frog muscle, and the viability of cysts of rotifers, etc. Most workers, however, consider these arguments invalid.

system. Furthermore, much of this research is vitiated by failure to specify concentrations, particularly of gases and vapors, and because of the use of single, or only very few, fireflies for each reagent tested. Obviously, we shall be able to utilize only small and isolated fragments from this literature in pursuing the problem outlined in the general introduction. Some of the remaining information, however, is of potential physiological use, though at present one can draw only the most obvious sorts of conclusions, such as that strong protein precipitants extinguish luminescence. The data have further value in showing that one cannot possibly regard the depression or stimulation of luminescence by a given agent as a specific effect, and that one cannot safely argue by analogy, in the sense of saying, for example, that because a capillary dilator increases luminescence, it does so by dilating the tracheoles.

Normal Types of Light-Emission. The difficulties in analyzing the various types of luminescence seen in different kinds of fireflies are increased by the fact that many workers have not distinguished between luminescent behavior in the field and that under laboratory conditions. There are four normal types of light-emission.

The Continuous Glow. This type of luminescence, in which the light is emitted as a glow of rather uniform intensity, usually continuing throughout life, is common in lower organisms such as bacteria and fungi, and is found in the larva of *Phengodes* and in the eggs and pupae of some fireflies. As far as I know, the glow occurs normally in only one kind of mature (adult?) firefly, the larviform female of *Phengodes*. Even here, it fluctuates moderately, following mechanical stimulation.

The Intermittent Glow. In this type of luminescence, the light is emitted as a relatively steady glow which lasts for seconds or minutes. Hasama (1942c) timed the spontaneous activity of a larva of *Luciola cruciata* for 15 consecutive glows and found that the average duration was 20 seconds (range 7 to 60) and the average interval between glows was also 20 (range 4 to 86). Comparable figures were also observed for the larvae of *Pyrocoelia rufa* and *Luciola lateralis* by Hasama (1942b and c), and for *Photuris pennsylvanica* by the writer. In intermittent glowing, the light ordinarily takes several seconds to increase from zero to maximum intensity, and a comparable period to disappear. In larvae of *Photuris pennsylvanica*, however, I have seen the light appear or disappear in approximately a second. Intermittent glowing is apparently under voluntary control, although quite often there seems to be no correlation between luminescence and either activity of the animal or external conditions. Such glows can often be intensified by mechanical stimulation of the animal. Intermittent glowing is characteristic of the genera *Phrixothrix*,

Diphotus, and probably *Lamproyris*,* and of the larvae of most fireflies. It also describes well the light-emission in *Pyrophorus*, although Harvey (1931) reported that, after a given glow has passed through its plateau phase and entered the decay phase, a rhythmic fluctuation, with a period of 0.8 to 2 seconds, and an amplitude variation of 25 per cent or more, can sometimes be observed "after the light has nearly subsided." Heine-mann (1872) apparently observed the same phenomenon. Harvey ascribed these fluctuations to direct nervous stimulation, although he stated that they might possibly be caused by "some muscular mechanism connected with local distribution of air. . ."

The Pulsation. Hasama (1942a, 1942b) described the light of *Pyrocoelia rufa* and *Luciola lateralis* as being emitted in pulses, averaging, respectively, 6 to 13 and 60 to 110 per minute, with slight sex differences. The pulses are usually fused, thus giving the effect of a continuous light fluctuating fairly regularly. There are also occasional periods of darkness. Schultze's description of a rhythmic ebb and flow in the light of the male of *Lamprohiza splendidula*, indicates that this species may belong here, too,† but no information accurate enough to justify including any other species is available. In regard to frequency, Gerretsen's *Luciola vitticollis* (60-120 per minute) resembles *Luciola lateralis*, as do several of the Oriental and East Indian forms mentioned in many of the extraordinary reports of synchronous flashing (Buck, 1938). However, no further data are available on their types of luminescence.

As will be seen shortly, some forms of flashing also show pulses, but, for the moment, these are considered to be basically different from "the pulsation", because (a) their frequency is very much higher, (b) they occur as part of a rigid, regularly repeated pattern, each burst being followed by several seconds of darkness, and (c) the increase and decrease of luminescence is abrupt. On the other hand, the pulsation may not prove to differ fundamentally from the intermittent glow, in view of Harvey's (1931) photocell string galvanometer studies on *Pyrophorus*. He found that the apparently steady bright plateau glow actually often fluctuates at a frequency of $2\frac{1}{2}$ to 5 cycles per second, though with an amplitude variation (5%) which is too small to be detected by the human eye. For the time being, however, the category is useful for those forms which are neither typical glowers nor typical flashers.

* It is extraordinary that so few of the early workers troubled to describe directly the type of light-emission of their material, or to differentiate between the emissions of different types of organs, such as the lateral tuberculate and the ventral organs of the female of *Lamprohiza splendidula*. Spallanzani, and Carus (1864) mention that the larval light of "*Lamproyris*" is continuous, but this is opposed by tenuous allusions here and there in the literature. For the emission type of the adult, we have only the indirect statements of Owajannikow (1868) that the light of the male of *Lamproyris noctiluca* "is intermittent, though some lighted for hours"; of Wielowiejski that "after the stopping of intense glow a weak shimmer is seen"; and of Bongardt that "I have never observed that the *Lamproyridae* can suspend their light suddenly". Belleme states that the female of *L. noctiluca* requires 12 seconds for control, whereas the larva can extinguish its light "suddenly" (2 to 3 seconds). This indicates that at least *Lamproyris* is of the intermittently glowing type.

† Dahlgren writes of the female of *Lamprohiza* as glowing continuously and the male as flashing, but it is not apparent that he observed either sex critically.

The Flash. This is the most familiar normal type of light-emission in American fireflies and is seen in many of the common lampyrids (e.g., *Photinus*, *Photuris*, *Luciola italica*?*). In its simplest form, the flash consists of a burst of light of much greater intensity and much shorter duration than occurs in a glow. Ordinary observations show that the light intensity rises abruptly from zero to a maximum and then declines abruptly again to zero, but the duration of the flash is ordinarily so short that no accurate idea of the phenomenon can be obtained with the naked eye.† Using sensitive photocells in combination with amplifiers and a string galvanometer or oscillograph. Brown and King (1931), Snell (1932) and Alexander have carefully studied the "normal" (i.e., laboratory) flashing of *Photinus pyralis* and *Photuris pennsylvanica*. These studies show, among other things, that the duration of the flash is of the order of 0.1 to 0.2 seconds and rather constant, intraspecifically; that the peak intensity and total amount of light emitted per flash are quite variable; and that the augmentation and decay phases are fairly symmetrical (FIGURE 41). The extensive photometric work of Coblenz established 1/400 candle as the light intensity of an average flash of *Photinus pyralis*.

The limitation of most recent experimental work to *Photinus pyralis*, which has a simple single flash, and *Photuris pennsylvanica*, which seems to have a single flash under laboratory conditions, tends to obscure the fact that many much more complex types of flash exist. McDermott (1917) summarized the characteristic flash types given during normal flight by nine American species. Most of these are single flashes of various durations and relative intensities. However, the male of *Pyraclomena lucifera* and both sexes of one variety of *Photuris pennsylvanica* emit flashes with multiple peaks, instead of the simple "normal curve" type of luminescence (FIGURE 41). These flashes (better, "coruscations" or "twinkles") can be construed either as due to an optical fusion of separate flashes occurring close together or as rapid fluctuations in peak intensity of a single flash. I have records similar to McDermott's of the flashing characteristics of about forty species of Jamaican fireflies, of which eight show a coruscating type of flash. These multiple flashes differ interspecifically in duration, and in the frequency of the individual peaks, and in some species occur in complex, but constant, combinations with single flashes. The maximum frequency of the oscillations in intensity during a coruscation is not known, but is certainly not much less than the critical flicker fusion frequency of the human retina (probably from 25-30 cycles per second under the conditions of observation). Lund's description of

* In *Luciola italica*, Emery described the "increase and decrease of the light in short regular intervals," and Verworn (1892) described the "rhythmic intermittency" of the light of both sexes in flight. The frequency was 60 to 80 per minute according to Verworn, and 80 to 100 per minute according to Peters (1841). Verworn mentioned a weak continuous glow, given while resting, and stated also that the light was not completely extinguished between flashes. This description fits pulsing as well as flashing, but Geipel's "strict rhythm of flashing and extinction" indicates that *L. italica* is a flashing type.

† Occasional individuals of flashing species show a very faint glow which persists between flashes.

"regular, rapid and numerous changes in intensity" in the flashing of two Jamaican species of *Photinus* almost certainly refers to such coruscations.

It is possible that even the apparently single flashes of some species may show finer cyclic oscillations similar to those discovered in *Pyrophorus* by Harvey (1931). In *Photinus xanthophotis catherinae*, which has a very large organ and a flash lasting about half a second, I occasionally saw, with peripheral vision, very slight and very rapid fluctuations. However, it is also possible that a subjective effect is involved in this, since it is well known that, with light of very low brightness, the eye tends to shift its retinal fixation point rapidly. Such an effect might also be the explanation of the "*ébranlement particulier*" of the "retina" which Bellesme reported in close observation of the light of *Lampyrus* in a darkroom.

In many flashing types of fireflies, light-emission is used in astonishing systems of mating signals in which the male flies about, flashing at regular intervals, while the female, usually at rest, flashes in response to his signals (Osten-Sacken, 1861; McDermott, 1910, 1911a, 1912, 1917; Mast, 1912; Buck, 1937b). These signal systems are remarkably precise in their time relations and differ characteristically in different species. In normal flight, the male produces his luminous unit (whether single flash, coruscation, or complex) at regular intervals, and this emission pattern is so characteristic that the males of each of the dozen or more species which may be active at night in the field at the same time can be identified reliably from observation of their flashing, alone. As might be expected, the frequency of flashing rises with temperature (Snyder and Snyder, 1920; Buck, 1937b).

In summary, these four types of light-emission can be interpreted as the results of four progressively more effective modes of control. The continuous glow is an indication of the inability of the animal to prevent the luminescent reaction, or even to change its rate materially. In the intermittent glow type, the organism can interrupt photogeny, but only slowly, and the plateau level of glow presumably represents the condition with no control operating. In the pulsation, a further refinement of control may be assumed to have developed, so that now the light can be made to fluctuate fairly rapidly. In the flash, finally, the control mechanism achieves its highest development, as indicated by its ability to bring about bursts of light of very quick accretion and decay, of short duration, and with complete extinction between even closely spaced flashes. Since, in most flashes, nothing resembling a "plateau" is reached, it is uncertain whether or not the peak of the flash represents maximum possible luminescence.

With the above hypotheses in mind, it is interesting to review these various types of light-emission in connection with the morphological studies already discussed. In the continuously glowing *Phengodes*, the

luminous cells lack any specialized tracheal supply and presumably obtain oxygen from the body fluid with which they are bathed. Tracheae are also absent in eggs, and presumably also in pupae, because of histolysis. In the organisms with intermittent glows, we find, exclusively, organs of Types 2 and 3, with a branched and tapering tracheal supply lacking end-cells. In the fireflies with the pulsating type of light-production, we find end-cells present, but with arborescent tracheal branching, as described for organs of Type 5. Finally, in the flashing type, we find the cylinder, the highest morphological development in the sense of precision and complexity of organization. Thus, there seems to be a striking correlation between the type of tracheal supply in the photogenic organ and the normal type of luminescence. In particular, the presence of end-cells seems to be associated with the ability to produce a flash or pulse. This has been pointed out previously by various workers on the Type 6 organ* and will be discussed further in connection with end-cell physiology. Whether or not the proposed distinction between the pulsation and the "true" flash will prove valid must await careful observation on further species having the Type 5 tracheal arrangement. At present, I am not able to suggest any very convincing anatomical reason why the Type 5 organ should be less able than the Type 6 to control luminescence abruptly. Control might be connected with the cylinder itself, but it might also be related to some other anatomical feature such as, for example, the differentiated zone of the photogenic cytoplasm, or tracheolar anastomosis, neither of which seems to have been recognized in firefly photogenic organs of Type 5.

Localization of Luminescence. Microscopic observation of the active photogenic organ, preferably in the living animal, provides the only acceptable evidence of where light-production is localized. A number of investigators have reported work of this sort. Except for Weitlaner (1909), no one, since Kölliker first demonstrated the two-layered structure of the organ, has doubted that the light is produced in the "photogenic" layer, although certain workers (e.g., Wielowiejski) thought that the reflector layer, too, was slightly luminescent. Almost certainly, this idea was fostered by contamination of the reflector layer with photogenic material or by diffusion of light from the underlying photogenic layer.

Spallanzani was probably the first to observe the surface of a glowing light organ. He reported having seen many tiny points of light. Kölliker and Schultze observed the same "minute sparks" in *Lamprohiza*, and Schultze, in addition, believed that the points corresponded to his newly demonstrated end-cells. Schultze is generally credited with the claim that the light is confined to the end-cells, but actually he referred to the light as "beginning" in the end-cells. Since he also suspected a connec-

* This is apparently what Hasema (1942c) was referring to in his statement that "many authors have ascribed the strongly rhythmic blinking of tropical fireflies to the concentric arrangement of the end-cells." (Translation.)

tion between nerves and "parenchyma" (photogenic) cells, Wielowiejski and Bongardt have argued that this implies his believing the photogenic cells to luminesce in addition. Bongardt and Lund described just such a spread of light from minute flashing points into a general glowing of the whole organ, and stated that the positions of the individual spots were constant, though their intensities were not. Bongardt also claimed that the number of end-cells, as determined from the points of reduction of osmic acid, far exceeds the number of lighting points, indicating that the end-cells are not the luminous points. This argument is not necessarily relevant, since not all the end-cells would need to be active at once.

The localization of luminescence has been much more extensively studied in the Type 6 organ than in *Lamprorhiza* and *Lampyrus*. Emery set the general pattern for all later workers when he described the surface of a glowing (not sparkling) *Luciola italica* organ as showing a pattern of luminous rings, the dark centers corresponding to the cylinders. The same sort of structure has been observed in *Photinus marginellus* (Townsend), *Photuris pennsylvanica* and three Jamaican Photini (Lund), and in *Photinus pyralis* and *Photuris pennsylvanica* (Alexander). Emery discovered, in addition, that the luminous rings were not uniform but showed spots which lit up and went out irregularly and were, he thought, constant in position. Emery is a little vague about the exact localization of these spots, but it appears that he believed the light to occur at the contact between the end-cells and the photogenic cells. Lund confirmed Emery's observations in all respects and added the finding that the end-cells did not luminesce. He also discovered that when an organ was glowing brightly enough so that the individual rings fused and made the whole intercylinder area luminescent, the brightest region was in the differential zone at the periphery of the photogenic cells. Alexander, in turn, confirmed Lund's report on the localization of the bright points of light at the bifurcations of the tracheoles, and on the fact that they fire repetitively and asynchronously. This rapid, irregular, scintillating or "spinhari-scope" type of local luminescence has also been observed by Wood (1939) in a firefly poisoned by a spider,* and by Kastle and McDermott (1910) and Alexander following a variety of treatments, of which strychnine injection was most effective. In addition, I have seen it following distilled water injections and cyanide vapor. Besides the glowing rings and sparkling points, "phosphorescent clouds" which sweep across the organ in waves have been described (Emery, Kastle and McDermott, Lund). According to Lund, they originate deep in the tissue.

The observations just described were used by Lund to support his claim concerning the enzymatic activity of the periphery of the tracheole, which has already been mentioned, and by Emery to further his idea that the photogenic material "secreted" in the light cells is transported to

* Steche (1908) observed normal intermittent flashing in a firefly apparently paralyzed by spider bite.

and burned at or in the end-cells. It should be kept firmly in mind, however, that no details of any kind can be made out in an organ which is flashing, or even exhibiting a bright glow, and that the conditions under which the luminous rings, scintillations, and cloudy waves are seen are distinctly abnormal. Moreover, it must be remembered that none of the findings have any necessary relevance for organs of Types 2 and 3, where end-cells and cylinders are lacking.

In contrast to light organs which contain end-cells, the organs of Types 2 and 3 show only a uniform and structureless glow. Dahlgren and others have pointed this out as characteristic of *Pyrophorus*, "the larvae of all Lampyrids", "the females of *Lamprorhiza splendidula* and all species of *Phengodes*". From personal observation, I can confirm Dahlgren's statement on *Pyrophorus* and the larva of *Photuris pennsylvanica*. I would expect the statement about *Lamprorhiza splendidula* to apply only to the lateral tuberculate organs. In *Phengodes*, I have described the light as coming from minute separate spots corresponding in number, size, and position to the huge oenocyte-like cells seen in histological preparations (FIGURE 15; Buck, 1946a).

The theories of Schultze and of Emery, which gave the end-cell a primary role in photogeny, were based on two observations: the presence of points of light in the general region of the end-cells in the living light organ, and the affinity of the end-cells for osmic acid vapor. They also involved the assumption that a high reducing power for osmium indicates a high affinity for oxygen. We have already seen that Lund denied that the end-cells actually luminesce, and that several workers have described the spread of luminescence throughout the photogenic cytoplasm. Nevertheless, observations on the living organ are not precise enough to rule out the possibility that the end-cells, or other inter-cylinder material, may light in addition to the photogenic cells. This is particularly relevant since so many reports speak of the luminescence beginning at the edge of the cylinder and since Lund himself admitted that the end-cells are often situated in little bays hollowed into the photogenic cytoplasm. The point about the site of reduction of osmium, which Emery regards as the "*experimentum crucis*," and which Lund also uses to bolster up his theory of the enzymatic activity of the tracheolar wall, has been dealt with on page 409. The particularly heavy deposit does not necessarily prove that there is anything unique about the end-cell, but merely that it stands at the first spot permeable enough to allow the vapor to escape. The slight penetration into the tracheoles would then be caused by the osmium being reduced as fast as it arrives at their proximal ends. Wielowiejski has argued, in addition, that a high affinity for oxygen does not necessarily identify the site of photogeny, and has made the interesting suggestion that the end-cell acts in a manner analogous to the red blood cell and "stores" oxygen. Wielowiejski's and Emery's

theories thus each provide that material, freed in the photogenic cells by nerve stimulation (the control), moves toward the end-cell, by a method not elaborated, and luminesces near it because the highest concentration of oxygen is found there. However, no evidence exists to show that any oxygen carrier is present in or around the end-cell, and in any event its activity would be an effect, rather than a cause, of luminescence.

Tracheolar Properties of Interest in Regard to Control of Luminescence. Creighton injected adrenalin into *Photuris pennsylvanica* and observed a bright glow. This, as we have seen, is the usual response to dozens of agents. On the basis of histological studies, he claimed that the adrenalin effect was not hormonal but was due to contraction of muscle fibers in the tracheal end-cell which enlarged the part of the tracheole within the end-cell, as postulated by Dahlgren. However, according to a personal communication cited by Alexander, it appears that Creighton's preparations showed a dilation not of the end-cell lumen but of the whole tracheole. We have, then, the possibility that luminescence may be controlled by an active or passive dilation and constriction of the tracheole, such as Gerretsen suggested as an alternative to end-cell activity.

Many have regarded the fact that the tracheoles do not dissolve in caustic solutions as proof that they are composed of chitin, although this has not been confirmed by more specific tests. If the walls of the tracheoles were composed purely of chitin, they would be unlikely to be very dilatable. However, as Richards (1947) has pointed out, protein often bulks large in cuticle and could satisfy most conditions for extensibility. It would be desirable to have better evidence of the chemical composition of the tracheoles, but color tests would not be conclusive, in my opinion, because of the difficulty in getting a strong enough color in a structure as thin as the tracheolar membrane. For many years, the absence of visible spiral thickenings in the walls of the tracheoles was regarded as strong evidence that they differ from the ordinary larger tracheae. However, Richards and Anderson (1942) have shown by electron microscopy that honeybee tracheoles have spiral thickenings at least down to a diameter of 0.2 micron. The presence of thickenings in a tracheole might reduce the extensibility of the wall, but would not prevent tracheol collapse, as Richards and Anderson have shown.* However, if Creighton's finding of tracheoles dilated by adrenalin is accepted, it indicates that the chitin content is low.

There is an opinion that tracheoles must differ in composition from tracheae because they are permeable to gases and liquids. This idea probably stems from Krogh's (1919) evidence that the diffusion rate of oxygen through chitin is only about one-thirtieth of that through water,

* References to additional literature on tracheoles will be found in Wigglesworth (1931 and 1939), and Richards & Anderson.

and about one-tenth of that through tissue. However, if it is recalled that the membrane of the tracheole is only between 0.005 and 0.01 micron thick (Richards and Anderson), it will be seen that simple transfer could go on readily even through chitin. Wigglesworth (1930) and others have, in fact, shown that tracheae are freely permeable to oxygen and nitrogen.

Hoskins 1940, has called attention to one possibly valid distinction between tracheae and tracheoles, which is that the former are as a rule distinctly hydrophobic, whereas the latter are often strongly hydrophilic. Even here, some confusion exists, since it has been reported that the tracheae are hydrophilic in newly moulted insects (Wigglesworth, 1938b) and, conversely, that oil which enters the tracheae eventually penetrates into the tracheoles (Hoskins).

It is almost universally held, either explicitly or implicitly, that the principal function of the tracheoles in the light organ is to conduct oxygen to the photogenic cells. It should be emphasized that, however reasonable this view may appear, it is only an assumption. Moreover, there has not even been a serious attempt to define, theoretically, the conditions under which oxygen conduction might take place. Among the more obvious items of needed information are data on whether transport occurs by diffusion or by flow, and whether the tracheoles contain air or liquid. A further discussion of theoretical aspects of these questions will be found in the section on end-cell physiology. For the present, we can conclude only that, if transport is by diffusion, the tracheole loses most of its meaning if it is assumed to be filled with liquid, because the diffusion of oxygen down a column of water is no faster than it would be in free water, and only about three times as fast as through dense connective tissue (Krogh). Only by containing air, in which oxygen diffuses thousands of times as fast as through water, could a tube confer any advantage in supplying oxygen. To be sure, if the tracheole were absolutely impermeable to oxygen for some of its length, access of oxygen to parts of the photogenic cytoplasm could be prevented or delayed even if the tracheole were liquid-filled, but it is hard to see what advantage would be conferred thereby and, moreover, we have already seen that impermeability to oxygen is extremely unlikely.

Another possibly significant point is Wigglesworth's (1930) observation that air bubbles, trapped between two columns of oil advancing toward each other in a trachea, are completely squeezed into solution, in the hemolymph or tissue surrounding the trachea, by the surface tension of the oil. I have seen the same thing happen in dipteran larvae immersed in kerosene. An astonishing feature of the process is its speed. The factors involved are too complex to permit one to say off-hand whether the same phenomenon would occur in tubules of the dimensions and structure of tracheoles, or, if so, whether it would be rapid enough to meet the requirements of photogenic control. However, the principle should be

kept in mind as a possible method of forcing oxygen into cells. If, for example, the tracheal end-cells were to "close", and if water moved up the tracheole proximally, it might drive some of the contained air into the photogenic tissue. This would be the reverse of the theory of osmotic control of luminescence to be discussed in a later section.

The Nervous System and Luminescence. A connection between the nervous system and luminescence has long been accepted by most investigators, on the basis of the anatomical evidence already discussed and because of a variety of experimental work. It was shown very early that decapitation, or cord section anterior to the organ, results in immediate cessation of voluntary flashing or glowing, though a dim constant luminescence may persist, or reappear some time later (Macaire, 1821; Peters, 1841; Verworn, 1892; Dubois; Prowazek, 1908; Lund, Williams). Likewise, luminescence is initiated or increased by stimulating the nerve cord* mechanically (*e.g.*, Heller, 1853; Verworn), or electrically (Macartney, 1810; Macaire; Todd, 1826; Joseph, 1854; Kölliker; Owsjannikow, 1868; Bellesme; Heinemann, 1886; Dubois; Fuchs, 1891; Steinach, 1908; Lund, Gerretsen, Perkins, 1931; Snell, 1932; Brown and King, Alexander), even after section proximal to the point of stimulation.† Direct electrical stimulation of the photogenic tissue likewise causes lighting. Nervous influence is also suggested by numerous observations that anesthesia suppresses voluntary control of luminescence,‡ and by the apparently stimulating effects of spider venom (Wood), various neurotoxins (Kuhnt), and DDT (page 75). In all these instances, however, direct action of the agent on the photogenic tissue, or on the tracheal system, cannot be excluded.

In contrast to the above work, Owsjannikow observed no diminution of light in isolated organs after 1½ hours' soaking in curare and strychnine, and concluded that the nervous system is not involved in luminescence. Bongardt repeated this experiment, using the entire animal, and reported no effect in 12 hours. Aside from the questions of penetration and of whether vertebrate poisons would be expected to act on insects (raised also by Bongardt), this conclusion involves a type of *non-sequitur* encountered frequently in the older work, and well illustrated also by Bongardt's argument that fireflies cannot stop their light suddenly, because dead ones continue to glow for 10 to 20 days. Owsjannikow and Bongardt thus failed to see that what happens in the injured or dead animal may be entirely irrelevant to the question of whether the nervous system, or anything else, permits voluntary control of luminescence in the normal living animal.

* Because of the minute dimensions involved, it is extremely likely that simultaneous stimulation of other tissues near the operated region was not excluded, particularly in the earlier work.

† Some of these papers report considerably more than the bare observation. They are interesting from the standpoint of comparative electrophysiology.

‡ However, luminescence itself may persist for long periods under conditions where voluntary neuro-muscular activity is suppressed or abolished entirely.

Harvey's (1931) photocell-string galvanometer records on the glowing of *Pyrophorus*, which have already been discussed, indicate that the light is under nervous control, particularly as Harvey was careful to exclude re-piratory and pulse movements. A better test could be made by recording simultaneously the action potential pattern of the ventral nerve cord, and the luminescence, particularly if done on several species with differing flash patterns. It may be of interest here to mention some other potentials associated with luminescence, though not necessarily with the nervous system. Many years ago, K  lliker obtained some "not quite constant" evidence that lighting fireflies deflect a "multiplier" (galvanometer) more than do non-luminescent ones. In view of K  lliker's primitive apparatus, his report does not call for extensive consideration. Recently, however, Hasama has reported that, in a number of Korean species, the light organ itself produces a monophasic action potential during activity. The luminous segments are electronegative to the non-luminous. In the larva, the potential pattern is continuous, and synchronous with light emission. In the adult, it exhibits rhythmic or cyclic fluctuations, the frequency of which corresponds satisfactorily with that of the pulsing of the light in *Pyrocoelia rufa* (13 per minute), but poorly in *Luciola lateralis* (48 per minute for the potential, 60 per minute minimum for the light). With the electrodes on two non-luminous segments, instead of on one luminous and one non-luminous, no potential difference was detected, thus indicating that muscle potentials, etc., are not involved.

A number of further lines of evidence indicate indirectly that the nervous system is concerned with the control of luminescence. For example, the rigid characteristic species flash-patterns already referred to, as well as the inherent diurnal rhythm of luminescent activity* (Allard, 1931; Perkins; Rau, 1932; Buck, 1937a), point to the existence of at least an involuntary center of nervous control. Indeed, Verworn, some sixty years ago, postulated in detail that normal luminescence is under absolute control of an automatic nervous center in one of the two most anterior ganglia of the cord. Though similar hypotheses have since been used by several writers in attempting to explain synchronous flashing (see Buck, 1938), work on the mating signals shows that a high degree of voluntary control can also be exercised. Briefly, these signal systems enable the female of one species, or an artificial light operated in a specific way, to attract males of the same species without attracting males of another species. Conversely, males of a given species signal only to a female of the same species (or to a properly operated artificial light) and ignore other males of their own species, both males and females of other species, and all improperly executed artificial signals. In *Photinus pyralis*,

* This periodicity may play an important and unsuspected part in certain experiments, since Malcoen (1938) and Alexander have claimed that it influences reactions as basic as the appearance of luminescence in high partial pressures of oxygen.

I have shown that the ability of the male to recognize the female depends on the fact that she always replies to his flash after a particular time interval (Buck, 1937b).

The fact of nervous influence, however, does not solve the problem of the control of luminescence, since the effect could either be direct (*i.e.*, stimulation of the photogenic cells), or indirect (*e.g.*, stimulation of the end-cells; oxygen regulation). On this question there is no dearth of opinions, pro and con, but valid empirical evidence has proved to be extraordinarily hard to obtain.

Evidence that oxygen regulation is the primary factor in control of luminescence is, of course, opposed to the idea of direct nervous stimulation of the photogenic cells. This evidence will be analyzed later in detail (page 441), but it may be said here that it is insufficient to exclude the possibility of direct nervous control.

Heinemann (1886) reported that electrical stimulation of nerves anterior to the abdominal photogenic organ of *Pyrophorus*, after all other tissues of the trunk had been transected, did not induce luminescence. From this he concluded that the nerves act by way of some intermediate tissue. However, since it was a negative type of experiment, and since Lund, in repeating it, obtained precisely the opposite result, it cannot be regarded as decisive. It is also difficult to reconcile with numerous reports of luminescence obtained by electrical stimulation of isolated abdomens of lampyrids, and with the experiments of Fuchs on localized stimulation of the photogenic organs and nerves of *Pyrophorus*.

Considerable evidence has been adduced in favor of direct nerve action. Lund's transection experiment has already been mentioned. However, Lund's acceptance, on the basis of this and other work, of "primary control of the organ . . . by nerves in direct connection with the photogenic tissue" is greatly weakened by a number of apparent contradictions in his presentation. For one thing, it appears that, in spite of denying oxygen a main role in control, he actually considered the end-cell to be of prime importance. For another, he emphasized the well-known fact that the photogenic tissue itself "is irritable and responds locally to mechanical stimuli", overlooking the probability that such disturbances also facilitate access of oxygen. Steinach, using induction shocks applied directly to the photogenic organs of decapitated specimens of *Lampyrus*, claimed to have demonstrated summation of subliminal stimuli for luminescence. He attributed the summation to a direct effect of the nerves on synthesis of substrate for luminescence, and rejected the alternative idea of simple neural summation, although his reasons for so doing are unconvincing. Bellesme also supported direct nervous stimulation of synthesis on the basis of an experiment in which he found that fireflies cut open in air luminesced sooner, if they had first been "stimulated" electrically in the absence of oxygen, than if they had not. However,

aside from the unavoidable variations in manipulation, the temporal variability in responses of fireflies under such conditions is ordinarily so large that a conclusion cannot be accepted until verified on a considerable number of specimens. Moreover, no allowance was made for the probable effects of the anoxia upon the nerves. Additional details concerning the work of Steinach and Bellesme will be found on pages 438 and 441. Work of Snell bearing on the same problem is considered on pages 442 and 443.

Perhaps the strongest evidence of direct nervous control of luminescence is furnished by the numerous experiments on electrical stimulation of *Pyrophorus* and of firefly larvae. Here, the finding of increased intensity of luminescence with increased intensity of stimulus is less easy to attribute to oxygen control, since these forms lack end-cells.

The problem of direct *versus* indirect nervous control of luminescence is thus unsolved, although there is much evidence in favor of the former alternative. Unfortunately, the effects of oxygen and nerve action seem inseparable, experimentally. Under abnormal laboratory conditions, it is clear that nerves are not essential for luminescence, since fireflies will glow under a variety of conditions incompatible with nerve action, or even with life, if oxygen is present. Nevertheless, as we shall see, there is no conclusive evidence that oxygen is ever limiting in normal flashing, and there are many indications that control is much more circuitous than by direct regulation of oxygen access to the photogenic tissue. A number of hypothetical mechanisms of intracellular control, such as might be set off by direct nervous stimulation, are discussed on pages 434 to 436. Moreover, it should be kept in mind that the fact that the experiments discussed are inconclusive, does not disprove direct nerve action. The interpretation of Bellesme and Steinach may yet turn out to be correct, even though it does not necessarily follow from their experiments.

THE CONTROL OF LUMINESCENCE

Historical. Before entering on the detailed discussion of modern work and theories on control of luminescence, it may be interesting to consider, briefly, a few of the older theories proposed during the long history of the study of fireflies, and now abandoned. Perhaps the oldest and most persistent idea was that luminescence is controlled in some way by "respiration", by which apparently was meant that the muscular respiratory movements pumped air into the photogenic organ (Joseph, 1854; Faraday, 1814; Carrara, 1836; Siebold, 1848; Leydig, 1857; Seaman; Heinemann, 1886; Watasé). Several of these observers maintained that the breathing movements are synchronous with light-emission. However, breathing movements could hardly be the only control, unless the light pulsated day and night or the insect stopped breathing by day, neither of which alternatives seems likely. Elaborations on the idea were pro-

posed by Carrara, who claimed to have found an air tube leading from the mouth to an abdominal bellows or vesicle; by Heinemann (1886), who envisaged the abdominal musculature as pumping air through the "hiatuses" in the photogenic layer of *Pyrophorus*; and by Seaman, who suggested that the spiracles were first closed and air was then forced into the organ by abdominal contraction. A serious stumbling block for these theories is the fact that most modern workers have been unable to observe any visible muscular movements coinciding with luminescence (Lund: Harvey, 1931; Hasama, 1942a). Likewise, Heinemann's theory has little support, since Geipel has shown that the musculature of *Pyrophorus* is in no way different from that of non-luminous elaterids, and since, as previously stated, the existence of the spaces in the photogenic layer is very dubious. The same objections apply to Dubois' theory, according to which not air but blood was supposed to be percolated through the photogenic organ of *Pyrophorus*. The circulatory system was also implicated by Carus, who maintained that the rhythm of luminescence corresponded to the pulse, and by Tozzetti, who suggested that the tracheoles carried part of the blood.

Another theory was to the effect that the light-emission was controlled by withdrawing the light organ into the interior of the abdomen, where it was concealed by other viscera (Carradori, 1797; Müller, 1805; Owsjannikow, 1864).

Deductions from Kinetics Analysis. By high-speed recording, Brown and King, Snell and Alexander obtained time-intensity curves of the flashing of *Photuris pennsylvanica* and *Photinus pyralis*. Snell pointed out that the form of these curves is determined by three variables: the number of photogenic units active, the degree of activity of each unit, and the degree to which each unit is stimulated.

If these curves represented the response of a single photogenic unit, or the simultaneous responses of all the photogenic units in the light-organ, their analysis could reveal valuable information on the luminescent reaction and its control mechanism. From the form of the accretion and decay phases, it might be possible to deduce, for example, the number of reactants involved in the rate-determining reaction and the number of units active; and to ascertain whether diffusion or "phase-boundary removal" (see page 436) brings the reactants together; whether or not the unit response is of the all-or-none type; whether the control mechanism "opens" by relaxation or contraction; etc. Brown and King, in fact, suggested a relation between the supposed logarithmic form of the decay phase of the flashing curve, and the similarly logarithmic form of the decay phase of the luciferin-luciferase reaction (*Cypridina*) *in vitro*. According to Snell, however, a logarithmic form is the exception rather than the rule. Snell found the durations of normal flashes very constant,

the intensities highly variable, and even the reaction velocities (slope of decay phase) different.

The two segments of the luminous organ of the male of *Photinus pyralis* contain about 6,000 cylinders, each of which encloses a minimum of 80 to 100 end-cells, which, in turn, give off twice that many tracheoles. The tracheoles supply an estimated total of 15,000 photogenic cells, each of which is in contact with two cylinders (FIGURES 13 and 31). In studying the glows produced by various agents, I have noticed many times that single cylinders may light as individual units—that is to say, the surface of the organ may show minute isolated “doughnuts” of light which glow on and off independently. We must allow, then, for at least 6,000 photogenic units. If we accept Lund’s and Alexander’s claims that the end-cell /region/ can flash independently of the photogenic cells, as in “scintillation,” the number must be increased to something like 600,000. In either case, there is a strong likelihood that the time-intensity curve for the flash of the whole organ represents not the result of an absolutely synchronous firing of all the units, but the statistical result of the firing of units slightly out of phase. This is supported by the suggestive resemblance of the time-intensity curve to the normal-distribution curve. If the curve is indeed a statistical one, we can draw the very important conclusion that the duration of the luminescence produced by a single photogenic unit must be far less than the 0.15 second average for the mass flash. We have no way of knowing what the duration of the flash of the individual luminescent unit (as distinguished from its glow) may be, but it can hardly be more than one-tenth of that of the collective flash. The importance of this inference is that it imposes much more severe conditions upon the control mechanism. We now require a mechanism capable of producing isolated flashes of light with durations of the order of 0.01 second, or less. The same conclusion is indicated by the observations previously reported on the repetitive or oscillating types of flash seen in some Jamaican fireflies and in *Photuris pennsylvanica* in the field, although, here, there remains the possibility that the coruscations could be produced by groups of units firing successively in relays.

Mechanisms of Intracellular Control. We have seen that there is no agreement on whether flashing is controlled primarily by the nervous system or by the oxygen supply system. There is also no agreement on whether these two systems operate directly, *i.e.*, on the photogenic cell, or indirectly, *i.e.*, on each other. It is therefore appropriate to consider what general types of mechanism within the photogenic cell might be available as a basis for assumed “direct” effects of nervous or oxygen stimulation.

The protozoan *Noctiluca* is described (Quatrefages, 1850; review in Harvey, 1940) as emitting a bright flash, consisting of the momentary lighting of a multitude of tiny sparks scattered through the proto-

plasm, each representing a granule of photogenic material. The flash is not repetitive or voluntary, in the sense of being initiated internally, but is a direct response to some environmental stimulus (osmotic, thermal, chemical, mechanical, etc.). The synchronous lighting of the cytoplasmic granules during the flash is presumably attributable to the fact that the stimulus reaches the cell through the ambient water and, hence, almost simultaneously at all points on the surface. The bare essentials for controlled luminescence thus seem to be, first, the presence of a chemiluminescent system in the cytoplasm; second, a source of the necessary raw materials (here obtained from the surrounding sea water); and, third, a stimulus which will set off the intracellular reaction.

Harvey (1940) has pointed to the fact that a single cell like *Noctiluca* can flash as showing that complicated physiological mechanisms, such as those in the firefly, are not necessary for the control of luminescence. He thus regards the response of *Noctiluca* as quite similar to the responses of other cells (e.g., muscle) to direct stimulation, and implies that the response of an individual photogenic cell of the firefly need not, intrinsically, be any more complex. According to Harvey's view, therefore, the nervous system would correspond to the environmental changes which stimulate *Noctiluca*, and, in the firefly, would represent the anatomical and physiological answer to the problem of controlling the responses of thousands of *Noctiluca*-like photogenic units. The tracheal system, likewise, would stand in the same relation to the photogenic tissue as the sea-water to *Noctiluca*, and would be an arrangement for attaining, throughout a massive tissue deep within a body, conditions of aqueous diffusion of oxygen comparable to those of a free cell suspended in aerated sea-water. We may, therefore, think of *Noctiluca* and the photogenic cell of the firefly as presumably equivalent in regard to their intracellular control mechanisms.

Before considering possible intracellular controls, however, it is well to emphasize that the nervous and tracheal systems of the firefly, subsidiary though they may be, have important influences on luminescence. They are, therefore, as much a part of the control problem as the intracellular activities, and can be investigated independently of the latter.

A number of intracellular control mechanisms have been proposed, all on largely speculative bases. Perhaps the simplest of these, which may be called the "reactant limitation hypothesis", postulates that luminescence is controlled by limiting one of the four fundamental components of the luminescent reaction. With respect to oxygen, this hypothesis is equivalent to direct external regulation, but in regard to the other three essential reactants, even our very limited knowledge of the chemistry and enzymology of luminescence is sufficient to suggest various intracellular processes which might limit light-emission. Various reports indicating control by substrate synthesis, or storage and release, are considered on pages 437 to 440.

A second intracellular control mechanism has been proposed by Harvey on several occasions, and may be called the phase-boundary hypothesis. This hypothesis provides that all the essential reactants are present in the cell at the same time, but are prevented from reacting with each other by phase-boundaries. When these boundaries break down, as might occur as a result of nervous stimulation of the cell, the reactants can come together and produce light. Phase-boundary phenomena are well known in physical chemistry, and there are a few suggestive physiological and biochemical analogues, but we are still far from having a clear idea of how potential reactants are segregated from each other in the living cell. In fact, phase-boundary changes may only be some subtle form of reactant limitation. A single test of the phase-boundary hypothesis has been made on fireflies, but, as described on pages 442 and 443, the results were not conclusive. The similarly inconclusive experiments of Bellesme and Steinach on direct nerve action (pages 432 and 437) can also be interpreted as consistent with phase-boundary control. For the present, however, the concept must be considered simply as a theory, albeit a reasonable and stimulating one.

A third hypothetical intracellular control mechanism is one which makes use of a possible competition for oxygen between respiration and luminescence. This hypothesis, which has a number of variants, and which has not been tested experimentally, will be discussed on page 448.

None of the above discussion of intracellular mechanisms bears on the validity of the commonly held view that nervous control is direct and the tracheal system merely a means for ensuring that adequate oxygen is always available for luminescence. We shall be able to judge this better after having considered the evidence on oxygen limitation.

In conclusion, on *a priori* grounds it appears that intracellular control mechanisms would have a considerable advantage over external control mechanisms (*e.g.*, tracheoles or end-cells) in regard to speed of action. This might be crucial in the control of high-frequency light-emissions, such as are seen in the coruscating types of flashing. On the other hand, by making the control of luminous intensity more or less independent of changes in external oxygen tension, doubt is cast on the generally accepted significance of certain anatomical features of the photogenic organ, particularly the end-cell.

Water as a Possible Limiting Factor in the Control of Luminescence. Some of the earliest experiments demonstrated that if a firefly or an extirpated organ is quickly and thoroughly dried; it can be preserved in a non-luminescent state for long periods and will glow again when moistened (Spallanzani, Carradori, Macaire, Kölliker, Carus; Owsjannikow, 1868; Dubois, Bongardt, Kastle and McDermott; Hasama, 1942a). However, it seems extremely unlikely that water lack is ever a direct factor in controlling light-emission in the living animal, since this would

appear to demand a high-frequency cyclic dehydration and rehydration of the photogenic cells, and since luminescence continues for a considerable time in minced organs, where dehydration must be severe. This, then, is a good example of a method of limiting the luminescent reaction *in vitro* (or at least in the absence of living protoplasm) which is not involved in the normal control of photogeny.

Luciferin and Luciferase as Possible Limiting Factors in the Control of Luminescence. In discussing various theories of photogenic control by limitation of reactants, it should be understood that the terms *photogenic material*, or *substrate*, could refer either to luciferin or luciferase, since there is no evidence which points specifically to either.

If either luciferin or luciferase were not in the luminous organ, the problem of control could be simplified. It was, in fact, a convenient feature of Dubois' control theory that the luciferase was brought to the organ by the blood which circulated through the interstices of the photogenic layer and there met the luciferin. In all modern speculations on luminescence control, however, it has tacitly been assumed that these two reactants are formed in the photogenic layer and remain there, and that control is therefore a cellular problem. Moreover, most writers have assumed that photogenic materials are usually stored in the photogenic organ in excess, and that synthesis of substrate has no role in rapid control of luminescence. This view is based on the aforementioned observations that dead, minced, or even dried and remoistened fireflies or organs will glow for many hours. Since, however, the enzyme luciferase seems to survive even drying, there is no *a priori* reason why synthetic or other enzymatic methods of releasing substrate might not also survive.

In considering luciferin or luciferase as a possible limiting factor in luminescence, it is necessary to distinguish clearly, as Bellesme and Gerretsen have done, between the reactions which form the luminous material and those in which luminescence takes place. This distinction has often been overlooked. It is also necessary to differentiate carefully between mechanisms which operate to keep luciferase or luciferin separated (both being present) and those which limit the rate of formation of one or the other. The phase-boundary control mechanism, based on the former hypothesis, has already been discussed (p. 432). The latter hypothesis, according to which the rate of synthesis of "photogenic material" in the photogenic cells may become limiting, has also been debated, though not always in a clearly defined form. Steinach made implicit use of the hypothesis when he reported that stronger electrical stimulation produces a stronger light because more "light-stuff" is formed. Bellesme supported the same hypothesis explicitly when he postulated that the photogenic cells secrete a gaseous product (phosphine!) which lights as soon as it comes in contact with air. On the basis of the rather dubious

claim that a thoroughly crushed glowworm does not glow (when it ought to, since its interior is exposed to air), Bellesme postulated, in addition, that no lighting material is stored in reserve. In further support of this, he reported that there was a latent period between stimulus of the animal and the beginning of luminescence, and that this decreased if the firefly was first stimulated electrically in the absence of oxygen, so as to build up a store of luminous material. Delay might very well be seen in glowing forms, where the build-up of luminescence is slow. but, as we shall see, it is more likely to have another explanation. Additional details of, and objections to, the experiments of Steinach and Bellesme have been presented on page 432.

The inference that storage is shown by the fact that a firefly induced to flash for prolonged periods eventually becomes unable to luminesce, is too fallacious for extended consideration.

The question of storage of photogenic material was also considered by Wielowiejski and Bongardt, though in a very circuitous fashion. Wielowiejski opposed the idea of storage of substrate on the grounds that otherwise it would be impossible to explain how fireflies are able to extinguish their light voluntarily. This idea was based on Wielowiejski's convictions that neither end-cells nor oxygen are directly concerned with the control of luminescence, and that it is inconceivable that the nervous system could affect cellular oxidations directly. To these assumptions Wielowiejski added the very dubious argument that the reason why an isolated organ can glow for a long time is that excess photogenic material is formed during the act of extirpation. Bongardt advanced what seems to be the diametrically opposed argument that photogenic material must be stored in excess, because fireflies are not able to extinguish their light suddenly. It appears, however, that both these workers had vaguely in mind the concept that luminescence continues as long as stored substrate remains, but that they differed in their ideas of how long luminescence continued and how quickly it could be terminated. Their accounts are further confused by failure to distinguish the luminescence of dead fireflies from that under normal control, and by the fact that their evidence against oxygen as a controlling factor is very equivocal.

Gerretsen's views on reactant limitation suffer from the same sort of ambiguous duality, since he assumed control by the end-cell (oxygen limitation) but, at the same time, postulated that stimulation produces a substance which emits light when it comes in contact with oxygen (control by substrate synthesis). However, Gerretsen did elaborate on the relation between synthesis and luminescence, in postulating, by analogy with Harvey's early chemical work, that the "oxidized light material" was reduced during the dark period between flashes and thus became ready for luminescence again. This idea had no experimental support until very recently, when Alexander, on the basis of his work on

the effects of high oxygen tensions, postulated that luciferin "is steadily accumulating in a potentially reactive state within the photogenic cells between flashes", and that under circumstances when oxygen ceases to be limiting (*i.e.*, is present in excess), "the rate of luciferin release . . . becomes the critical factor for luminescence." Alexander, however, considered luciferin release of importance only in the hyperoxic glow and not in the control of flashing. He was unable to define precisely what should be understood by "luciferin accumulation" and "luciferin release." If either process involved synthesis, it would be hard to reconcile with continuing luminescence after death. On the other hand, if "release" were to mean a sort of reduction*, depolymerization, or hydrolysis of stored reserve, Alexander's results might be useful in interpreting long-sustained luminescences. It would, of course, have to be assumed that the "release" could continue in fireflies dried and remoistened, and that sufficient luciferin could "accumulate" prior to death to last the required time.

Space does not permit a detailed analysis of the data from which Alexander drew his conclusion. There were, however, a number of lines of evidence which fitted together consistently. On the other hand, to deal with the control of ordinary flashes and glows, Alexander adopted intact the end-cell theory of Dahlgren, which will be discussed later. It is, therefore, a possible weakness in Alexander's work that two entirely different mechanisms have to be invoked to account for all the luminescent phenomena observable in the firefly. All of Alexander's data, moreover, can be explained consistently by the modified mechanical end-cell theory which I shall present in the last section of this paper. Again, space does not permit a point-by-point comparison of the two schemes at this time. In any case, no final decision could be reached as to their respective merits, since both fit the available evidence satisfactorily. However, I consider it an advantage of the mechanical end-cell theory that it explains both normal flashing and glowing, and also the various abnormal types of luminescence.

In summary, then, there are two views in regard to the possibility that luciferin (or luciferase) could be the limiting factor in the control of luminescence. By far the most popular view is that a large excess of stored material is always present in the photogenic organ. This is supported by the common observation of long sustained luminescences, particularly in dead animals. The other view is that the substrate for luminescence is being synthesized constantly, and that there is never enough of it on hand for more than one flash. As we have seen, the evidence for this view, in the form stated, is not strong. If, on the other hand, the limitation is thought of in terms of luciferin *release*, as suggested

* See remarks at the end of the section on the reflector layer (page 408).

by Alexander, rather than luciferin *synthesis*, there is a possibility of reconciling the two views, in the sense that stored reserves could exist. It should be kept in mind, however, that it would still be necessary to exclude the possibility of oxygen limitation, and to show that luciferin accumulation (*synthesis*) and release could explain all normal luminescent phenomena.

Oxygen as a Possible Limiting Factor in the Control of Luminescence.* Although depriving fireflies of oxygen in various ways has been perhaps the most popular single experiment on bioluminescence, the instances are rare when this has been performed or described with sufficient precision to be of much value. To the familiar difficulties of failure to distinguish abnormal from normal luminescence, and effects on control systems from effects on luminescence *per se*, there has been added the failure to use strictly oxygen-free gases and leak-proof exposure chambers. In addition, sufficient attention often has not been paid to the facts that many common fireflies rarely flash or glow spontaneously in captivity, and that practically all fireflies are normally non-luminous by day (many observers) and in bright light (Buck, 1937a, and others). Hence, extinction of light in a given gas, or failure to luminesce, is not in itself necessarily proof that luminescence cannot occur in that gas.

In spite of these experimental deficiencies, there is no possible doubt that oxygen has a profound role in photogeny in fireflies. Luminescence has been reported to be reversibly extinguished (eventually), or not to develop, in pure N_2 , H_2 , or CO_2 (Forster, 1783; Spallanzani; Grotthuss, 1807; Macaire; Matteucci, 1843; Joseph; Owsjannikow, 1864; Bellesme, Dubois, Watasé, Bongardt, Townsend, Shafer, 1911; Kastle and McDermott, Creighton, Snell, Emerson, 1935; Hasama, 1942a; Alexander; Buck, 1946b). Snell's results are particularly valuable, since he was able to show that luminescence is possible below 4 mm. of O_2 . This astonishingly low oxygen requirement suffices to explain reports of failure to get extinction of light with N_2 , H_2 or CO_2 (Davy, 1810; Macartney, Bongardt, Kastle and McDermott). It also accounts for continued luminescence under oil (Carradori) or boiled water (Dubois), although utilization of air trapped in the tracheae may also have been involved (see p. 444). It also suggests that a control mechanism which operated by regulating oxygen would have to be of extraordinary efficiency. Additional indication of the necessity for oxygen is furnished by the observation that luminescence disappears in an evacuated space (Macaire, Owsjannikow, Dubois, Bongardt, Snell, Alexander). Here, the failures of Carradori, and of Kastle and McDermott, can be ascribed to insuffi-

* In this paper, "anaerobic" and "anoxic" will be used in their strict meanings of absence of oxygen, while "hypoxic" will be used for tensions (partial pressures) of oxygen (pO_2) which are abnormally low.

cient pressure reduction, as can Dubois' claim that a dried organ glows in a vacuum if moistened.*

Macaire made the interesting observation that fireflies made non-luminous in a vacuum cannot be induced to luminesce, by subjection to either heat or electricity. Similar observations were made later by Bellesme and by Knoche (1910) in regard to electrical stimulation of fireflies rendered non-luminous in irrespirable gases. These experiments have been widely interpreted as proving, first, that there can be no direct nervous stimulation of the photogenic cell; and, second, that the normal control of luminescence is by way of oxygen. Since these are very important conclusions, it is necessary to emphasize that the abovementioned experiments are entirely vitiated by the probability of an anoxic effect upon the nerves themselves. In this connection, it is interesting to note that the voluntary control mechanism is usually inactivated by oxygen lack before luminescence is abolished (see pp. 444 *et seq.*). Moreover, in regard to the relevance of these experiments to direct nerve action, there is no reason to expect that luminescence could occur in the absence of oxygen.

Arnold (1881) claimed that electrical stimulation induced *L. noctiluca* to light in "absolutely oxygen-free hydrogen" (as judged by the disappearance of the glow of stick phosphorus in the same chamber). However, since (a) he stimulated only within five minutes of the darkening of the phosphorus, (b) the glow persisted for a time after the current was broken, and (c) he could get only a single response, the glowing was probably made possible by air still remaining in the interior of the tracheal system, as suggested by Edwards (1863).

Experiments with increased oxygen have also been tried, but with conflicting results. Augmented luminescence has been observed by Forster, Spallanzani, Matteucci, Owsjannikow, Bellesme, Severn (1881), Kuhn, and Emerson and Emerson (1941), no change by Hermbstadt (1808), Davy, Kölliker, Macartney, and Dubois; and an actual decrease in emission by Macaire, Bongardt, and Hasama. Bongardt and Hasama claimed this last result to be a purely secondary one caused by the inhibitory effect of the actual gas current, because a stream of ordinary air likewise inhibited luminescence. However, their contention that this is also the explanation of the effects of N_2 , H_2 , and CO_2 , is logically unsound. Moreover, Knoche, and Höllrigl, in repeating Bongardt's work, showed that when precautions were taken to remove O_2 from CO_2 and H_2 completely, no luminescence could be elicited in *Lampyris*. Knoche also found a stimulating rather than depressing effect of gas flow. I have reported a similar effect of gentle air-currents on the flashing of fireflies in flight in the laboratory (Buck, 1937a). Severn reported that air cur-

*There are, as a matter of fact, two possible objections to results obtained with vacuum. First, if evaporation of water were fast enough, the cooling effect on the firefly might in itself inhibit luminescence. Second, if sufficient water were lost, lighting would be limited by water, rather than by oxygen. Probably neither objection is serious in short-term experiments with moderate rates of evacuation.

rents had no effect on luminescence. However, since inhibitory effects of strong air currents have been observed in the field by Geipel, and by myself, and in the laboratory by Dubois, a reflex mechanical inhibition of lighting, in accord with Bongardt's observation, is a possible source of error to be kept in mind in all work on gases, since their effects are almost always tested in streams. The effect of elevated oxygen tension is also seen in the increased luminescence obtained with high air pressure (Dubois, Lund, Alexander). According to Alexander, this corresponds with the effect obtained with nitrogen-oxygen mixtures at atmospheric pressure but with equivalent partial pressures of oxygen. Heinemann (1886) may have been dealing with the same phenomenon when he found that blowing air through a thoracic spiracle of *Pyrophorus* increased the light, although mechanical stimulation is a more likely explanation. None of the above effects bears on whether or not the oxygen effect is direct or indirect, since the experimental conditions may well have been stimulating to nerves as well as to photogenic tissue.

Kastle and McDermott, Snell, Maloeuf (1938), and Alexander, in work on the effect of increased oxygen on *Photinus pyralis* and *Photuris pennsylvanica*, showed that these fireflies may develop a steady glow, the mechanism of which will be discussed later ("hyperoxic glow").

Another line of inquiry which bears on oxygen limitation is one undertaken by Snell, at the suggestion of Harvey. Snell attempted to ascertain whether the nervous control of luminescence is direct or indirect, by recording flashes of *Photuris pennsylvanica* in different oxygen tensions. The analysis was based on two assumptions: first, that the actual luminescent reaction is independent of pO_2 above about 2 mm., as Shoup (1929) had found to be true in luminous bacteria; and second, that the control mechanism opens to the same extent and for the same period each time it is stimulated. It was then argued that, if the amount of light in the flashes were independent of oxygen tension over a wide range, it would indicate that luminescence was controlled by "removal of a phase boundary between reactants" in the photogenic cytoplasm, due to direct nervous stimulation. Dependence on oxygen, on the other hand, would indicate that an end-cell valve was operating, because a given response would admit less oxygen at low tensions and thus support less total light-emission. Snell did not actually compare total emissions, but only the maximum intensities of the flashes at various oxygen tensions. He found that there is apparently a progressive, though moderate, decrease in peak intensity between 150 and about 20 mm. oxygen. From this, he concluded that luminescence is controlled by regulation of oxygen by the end-cells.

It seems to me that Snell's conclusion is not justified. In the first place, there is no reliable evidence that the end-cell is the actual control mechanism. Secondly, since the constancy of operation of the control

mechanism is a pure assumption, it could as well be argued that the results were caused by a slight progressive change in the control mechanism, such as might be induced in the nervous system by the changing pO_2 . As a matter of fact, since Snell observed only about a 40 per cent decrease in average maximum intensity over a 7-fold change in pO_2 , there must have been very considerable compensatory changes in the response of the "end-cells", if luminescence *per se* is to be considered as having been influenced by oxygen in the range from 150 to 20 mm. In general, then, the only conclusion justified from this work is that the intensity of luminescence varies with oxygen tension in the range explored. Direct nerve action is by no means excluded.

In summary, most of the careful work agrees in making oxygen essential for continued luminescence. Under various experimental conditions, oxygen may be the limiting factor in the control of photogeny, but there is no direct evidence that oxygen is ever limiting in normal flashing. Nevertheless, there is sufficient circumstantial evidence of normal control by oxygen to justify the additional inquiries described on the following pages. Thus far, the evidence has not permitted a decision as to whether the presumed control comes about by an actual limitation of oxygen as a reactant, or secondarily by the effect of oxygen limitation on some other mechanism such as the nervous system.

The Spiracular Factor in Luminescence Control. Since the spiracles guard the primary site of entry of oxygen into the tracheal system, and since, in many insects, they are known to be efficient in barring access of gases or vapors (Wigglesworth, 1939; Hoskins), their possible influence on luminescence in the firefly invites investigation. Maloeuf and Alexander mentioned the spiracles briefly in connection with their theories, but apparently they made no observations. On the other hand, Wielowiejski, Lund, and Gerretsen all argued that no control located in the large tracheae could account for the rapidity with which a flash starts and stops, because of the time required to exhaust the oxygen in the tracheal system distal to the valve. In agreement with this view, I found no correlation between the state of the spiracles and the time of occurrence or characteristics of normal or electrically induced flashes in *Photinus pyralis* (Buck, 1946b).

In the same note, I reported investigations on the influence of the spiracles on glows produced by anoxia, ether, cyanide, distilled water, and adrenalin. Injected water caused immediate spiracular closure in both normal fireflies and those glowing from ether or cyanide, and subsequent cessation of glow in the latter. With all the other treatments, the spiracles opened before glowing began, and in anoxia they closed after the glow ceased. They usually also closed at about the time the glow ceased in ether or cyanide vapor, or with injected adrenalin, but not infrequently the glow continued for a few minutes after the spiracles had

closed. This was construed as showing that an "internal control" was still "open." Calculations based on measurements of the tracheae immediately associated with the light organ showed that a sufficient volume of air is trapped in and close to the light organ to support luminescence for several minutes, if the oxygen requirements of the light organ are comparable to those of ordinary tissues.

There is, apparently, a further stage of spiracular opening following the secondary closure, because dead or moribund fireflies usually have their spiracles open, whether glowing or not.

From this work, it was concluded that, although the spiracles and "internal control" often operate simultaneously, the spiracles do not ordinarily exercise any direct control over induced glows in flashing types of fireflies. However, the calculations indicate that they can terminate glowing eventually, and I think it not unlikely that they are important in luminescence control in normally glowing types of fireflies such as *Diphotus*. I attempted to investigate this question in the larva of *Photuris pennsylvanica*, but found the spiracles too small.

As argued in regard to breathing movements, if the spiracles were the only means of oxygen control for the organism, luminescence would be expected to occur by day, since the spiracles would presumably have to open to meet respiratory needs. This would not agree with the usual reports that fireflies are non-luminous by day. However, since fireflies are also inactive by day, it is possible that their general metabolism falls so low that the spiracles would need to be opened only very infrequently. Under such circumstances, even if luminescence occurred when the spiracles opened, it might escape observation.

The Internal Control of Glowing. A large number of agents and treatments have been discovered which cause glowing in Type 6 fireflies (Verworn, Kastle and McDermott, Gerretsen, Creighton, Snell, Emerson, Emerson and Emerson, Alexander; Seifter, 1945; etc.). Of these, hypoxia and anesthetic vapors have proved to be particularly useful in studying the operation of the control mechanism, and from their use a number of conclusions have been drawn concerning the tracheal end-cell. Since none of this work actually bears on the anatomical identity of the regulatory mechanism, I shall use the noncommittal term, *internal control*.

Verworn, and also Gerretsen, studied the effect of strong chloroform vapor on *Luciola italica* and *Luciola vittata*, respectively, forms which apparently give frequent spontaneous flashes under laboratory conditions, in contrast to the rare flashing of our American *Photinus pyralis*. They observed three stages of chloroform effect: (1) reversible inhibition of flashing, with immobilization, and sometimes accompanied by a dull glow; (2) irreversible bright glowing; (3) irreversible extinction. Stage 1 was ascribed by Verworn to nerve narcosis, and by Gerretsen to transient contraction of the end-cells. Stage 2 was assigned by Verworn to direct

chemical stimulation of the nerves and photogenic tissue, and by Gerretsen to relaxation of the end-cells. Stage 3 was attributed by Verworn to exhaustion of the stored photogenic material, due to the luminescence in the second stage, and by Gerretsen to permanent damage to enzymes. Verworn's opinion in regard to Stage 3 is weakened by Gerretsen's observation that the dead fireflies in Stage 2 sometime glowed for hours if removed from the chloroform vapor. The idea of some sort of irreversible chemical damage to the luminescent system is supported also by my observations on *Photuris pennsylvanica*. In this species, I found that light organs from specimens rendered non-luminous by an hour's exposure to saturated ether vapor, would not glow even if minced in fresh air, a treatment which ordinarily suffices to cause glowing even in moribund animals. The above idea is also supported indirectly by Emerson's work with known concentrations of ether, in which he found that irreversible damage to the luminescent system results when lethal concentrations are reached.

Either because they used saturated vapor or because chloroform is rather toxic, Verworn and Gerretsen missed some important intermediate stages of anesthesia which can be demonstrated in gradual hypoxia and with gradually increasing concentrations of ether. Thus, Emerson, and also Buck (1946b) have shown that bright early ether glows are readily reversible. The same is true for glows produced by low oxygen (Bongardt, Kastle and McDermott, Snell, Alexander; Butck, 1946b).

Snell studied the effect of low oxygen tension carefully, and showed not only that partial pressures below 4 mm. induce a steady dull hypoxic ("anoxic") glow* but also that, if the oxygen tension is suddenly raised from below 4 mm. to 30 mm. or above, the glow brightens suddenly, and usually markedly, and then quickly subsides to zero ("pseudoflash"). Snell interpreted the hypoxic glow as due to entry of oxygen through inactivated end-cells, and the pseudoflash as an extra uncontrolled luminescence which is terminated by recovery and closure of the control mechanism (end-cell valve).†

A further refinement in "internal control" action was disclosed by my finding (Buck, 1946b) that with very gradually decreasing pO_2 a stage is reached where an hypoxic glow has not yet developed, but in which a pseudoflash can be induced. To explain this phenomenon, I postulated that the "aperture" of the internal control had enlarged enough to permit luminescence at high pO_2 (pseudoflash), but still not enough to permit it at low. Since luminescence (hypoxic glow) does develop at oxygen

* The hypoxic glow is ordinarily produced by using pure N_2 , H_2 or CO_2 , but Bongardt, Snell, and Alexander showed that it occurs also in atmospheric air if the total pressure is lowered enough to decrease pO_2 below 4 mm.

† The pseudoflash has been interpreted by Alexander in terms of the Wigglesworth-Maloeuf osmotic theory, which will be discussed later. It might also represent the oxidation of luciferin that had accumulated during the hypoxic period, as has been shown to occur in the "flash" of luminous bacteria. However, in a number of experiments with *Photinus pyralis*, I found no correlation between the durations of pseudoflashes and the durations of the preceding anoxic periods.

tensions still lower than in the above stage, I concluded that the aperture of the internal control enlarges faster than the pO_2 decreases. In other words, the internal control overcompensates in its response to decreasing oxygen tension. These facts enable us to make the further deductions that luminescence requires the entry of a certain minimum number of oxygen molecules per unit time, and that this can be achieved either by the entry of air higher in oxygen through a smaller aperture, or by the entry of air lower in oxygen through a larger aperture. This implies, furthermore, that the response of the internal control is a graded, rather than all-or-none phenomenon.

Further information on the operation of the "internal control" is contained in a number of observations bearing on whether or not flashing and glowing are controlled by the same mechanism. It has been reported generally that normal flashing does not occur under anesthesia, except occasionally at the start of exposure, before glowing has developed to full intensity. Unfortunately, the evidence is not clear-cut on whether or not flashing can be elicited artificially during glowing. Although his account is very ambiguous, Gerretsen apparently believed that luminescence could be affected by electrical shock during his Stage 2 (glowing) of chloroform narcosis. On the other hand, Snell (1932) and Alexander found that normal flashing is inhibited during the hypoxic glow. Furthermore, Alexander was unable to induce flashing during hypoxic glowing, by electrical treatment. These results have been regarded by Alexander, and others, as showing that the flash control cannot operate during glowing, or in other words, that flashing and glowing are independent phenomena. However, none of this work circumvents the objection that that hypoxia and anesthesia (like anoxia) undoubtedly abolish the normal functioning of the flash control mechanism. Furthermore, the failure to obtain flashing with hypoxia might have been due simply to the very low pO_2 , rather than to a necessary difference in the control mechanisms for flashing and glowing. In other words, luminescence may already have been maximal for the existing pO_2 .

Snell's work with low oxygen is particularly germane with regard to the distinction between glow and flash. He found that, below about 20 mm. oxygen tension, the duration of the flash lengthened progressively and the peak intensity decreased until, at 4 mm., the steady hypoxic glow resulted. The region between 20 and 4 mm. oxygen tension, therefore, is a transition territory in which it is hardly possible to distinguish flash from glow.

An additional point of importance concerns the normal "neuromuscular" state of the internal control mechanism. The fact that fireflies moribund from inanition (or those which fail to recover from moderate anesthesia) often exhibit a steady, lasting glow, indicates that in its relaxed position the "valve" is open, and that energy expenditure is required to keep it closed. The same conclusion is indicated by the work of Ger-

retsen and of Emerson on anesthetics, and by the work on the effects of oxygen, if the assumption is made that the internal control relaxes (opens) in low oxygen and contracts (shuts) in high (Snell, Alexander; Buck, 1946b). As a matter of fact, adrenalin, cyanide, and all the other glow-inducing agents can be imagined to have an analogous effect. The open position in hypoxia, anoxia, anesthesia, and death is compatible with an assumption that energy expenditure is required to keep the "valve" in the closed (tonic), or non-luminescent, condition. This point will be discussed further on page 450.

A further interesting point was discovered by Alexander in his extension of Snell's work on the effects of high oxygen tensions. Although there is not space here to give the evidence in detail, Alexander was led to conclude that the glow produced by high oxygen is due to oxygen leaking directly through the cytoplasm, thus by-passing the internal control.

In experiments on glows produced by ether, carbon dioxide, and adrenalin, I found the responses of *Photuris pennsylvanica* larvae similar to those of the adult *Photinus pyralis*. Since the larva lacks end-cells, it can be concluded that end-cells are not necessarily involved in the control of glow in adult fireflies. However, they might still be essential to flash control, since none of the work discussed in this section really touches that point.

Theoretical Mechanisms of Luminescence Control by Oxygen Limitation:

INTRODUCTION. In this section, the mechanisms of oxygen control which have been proposed in sufficient detail will be analyzed in the light of the anatomical and physiological evidence discussed, and according to certain theoretical considerations. A review of the anatomical path which oxygen must follow in its journey to the photogenic cells, suggests the following sites at which the passage of oxygen might be interrupted: the spiracle, the end-cell, the tracheole, the differentiated zone, the membrane of the photogenic cell, and the photogenic cytoplasm. The spiracle has already been considered and found not to be concerned in the control of flashing. What little information exists on the differentiated zone of the photogenic cytoplasm, and on the possibility of phase-boundary changes in the cytoplasm, has been presented and need not be reconsidered. The membrane of the photogenic cell is theoretically the most logical site of all for the operation of an efficient control, but since Harvey (1922) has shown that a number of cells are very permeable to oxygen, there is no reason to expect that control by changes in permeability to oxygen is likely.* We are reduced, therefore, to the tracheoles and the end-cells as possible agents in oxygen control.

* There is every reason to believe that this conclusion is valid generally, but Harvey's work actually concerned permeability under abnormal conditions (recovery from anoxia).

The discussion will be mainly in terms of the Type 6 organ, since it has been the subject of nearly all the modern physiological work. However, this should not justify any generalizations, either pro or con, concerning any particular theory. Thus, it seems certain from the time relations, if from nothing else, that a firefly with an intermittently glowing type of organ will require a very much less precise and intricate control mechanism than one of Type 6.

COMPETITION FOR OXYGEN, IN RELATION TO CONTROL. One question which may have an important bearing on control is whether or not there is competition for oxygen between respiration and luminescence. This possibility exists because both systems presumably draw their oxygen from a common intracellular "pool".*

There would be potential competition if, during luminescence, the oxygen supply fell below that necessary to support both luminescence and basal respiration. There are few data which bear, even indirectly, on what occurs in these abnormal circumstances. Harvey (1922) found that, in *Chaetopterus* sealed in water, luminescence disappeared long before spontaneous muscular activity. However this may not indicate that luminescence is more sensitive to oxygen lack than is respiration, but only that the oxygen uptake necessary to complete the enzymatic reactions in muscle can be long delayed, whereas those in luminescence cannot. Moreover, it is possible that the effect was due to accumulation of carbon dioxide, rather than to deprivation of oxygen. Shoup found that respiration† of luminous bacteria began to fall off when pO_2 had been reduced below 23 mm., whereas luminous intensity was unaffected until pO_2 reached 2 mm., at which point respiration was only 50 per cent of normal. If, as seems reasonable, the intensity of luminescence is proportional to the oxygen consumption of the luminescent reaction, these results suggest that luminescence may be more successful in obtaining oxygen from a deficient supply than is respiration. It is, however, not certain that this conclusion is applicable to fireflies.

If, as Shoup's results suggest, luminescence has a competitive advantage over respiration under conditions of restricted oxygen, it appears unlikely that luminescence could be suppressed by respiration under normal circumstances, where sufficient oxygen for respiration is always present. However, since Creighton has proposed such a mechanism, it will be worthwhile to analyze it in some detail, particularly as the analysis points to a possible experimental test.

* Recent work on luminous bacteria indicates that, although respiration and luminescence show considerably different responses to certain stimulating and inhibiting agents (e.g., potassium cyanide and urethane), they utilize parallel enzymatic pathways. With increased oxygen, for example, proportionately more oxygen goes through the luminescent system. (See Harvey, 1940, 1941, and Johnson et al.)

† Shoup used "respiration" as synonymous with total oxygen uptake, but in the present paper it is used in the restricted sense of oxygen uptake other than that required for luminescence. In practice, the two measures will probably be nearly identical, since Shoup's work, and that of Snell on the hypoxic glow of the firefly, indicate that the oxygen requirement of luminescence is very small in comparison with that of respiration.

In view of Shoup's work, respiration could deprive luminescence of oxygen only under one of the following conditions: (a) the oxygen requirements of respiration are actually lower than those of luminescence, and the photogenic tissue is maintained nearly anaerobic, at least during the dark periods; (b) respiratory activity is localized in a cortex surrounding the actual photogenic cytoplasm, so that oxygen diffusing in it can be "filtered" out before reaching the other reactions of luminescence; (c) the intracellular oxygen transport system channels oxygen preferentially into respiration (at normal pO_2). The first possibility is rendered very unlikely by the known low oxygen requirement of luminescence. The second possibility certainly appears unreasonable on general grounds. However, in its light, Dahlgren's speculations on the "oxygen-insulating" properties of the differentiated layer of the photogenic cytoplasm (see p. 413) assume a new and provocative significance, and perhaps deserve further consideration. The third condition is one which, by analogy with known systems, is possible, but which would not be expected to permit absolute extinction of luminescence,* since such "channelings" are usually questions of relative rates of utilization, rather than absolute exclusion.

Since the idea of competition seems not impossible, an enumeration of its nature and consequences is in order. According to Creighton's hypothesis, basal respiration normally uses all the oxygen available and, hence, prevents visible luminescence (dark period). If, however, additional oxygen is made available (*e.g.*, by end-cell contraction), photogeny may begin (luminescent period). There is, however, an alternative method by which competition could operate. According to this second hypothesis, the oxygen supply remains relatively constant, but is usually all appropriated by respiration, which proceeds at a rate higher than basal (dark period). If, however, the respiratory rate falls, oxygen is available for the luminescent reaction. Both hypotheses, it will be seen, are compatible with the fact that respiration can be maintained without permitting photogeny at "inappropriate" times. This is particularly pertinent in view of the very small pO_2 required for luminescence. Both hypotheses also are in harmony with the expectation that respiration is diminished or absent in moribund or dead fireflies, where luminescence is often continuous. Assuming that prolonged anesthesia depresses respiration, both hypotheses also explain glowing in narcosis. Neither hypothesis accounts for either hyperoxic or hypoxic glowing, without further assumptions which are too detailed to consider here.

Theoretically, a decision as to which mechanism (if either) is operating could be made by comparing the oxygen uptakes of a tissue in the dark and in the luminescent states. According to the first hypothesis, oxygen consumption should be higher during luminescence than during the dark

* In view of the extreme sensitivity of the human eye, it is likely that luminescence really is zero during the apparently "dark" period, rather than continuing at a low rate.

period; according to the second, probably lower.* Unfortunately, as already discussed, no direct data are available.† However, it will be instructive to pursue some of the consequences of the two hypotheses a little further.

The first hypothesis, according to which respiration is lowest during the dark period, fits better the traditional idea that control is achieved by limitation of the external oxygen supply. It agrees, for example, with the expectation that luminescence involves increased oxygen and that nervous stimulation induces luminescence by inducing an increase in that supply. Furthermore, a linear relation between luminescence and oxygen tension, at least over part of the range, would be expected on the basis of the first hypothesis, if the oxygen uptake of luminescence is linear with oxygen tension. No relevant work has been done on glowing forms, which would be the most favorable for the purpose; however, as we have seen, Snell's data indicated that peak flash intensity decreases as the partial pressure of oxygen decreases from atmospheric. On the other hand, in several organisms (notably bacteria), luminescence has been found to be independent of oxygen over very wide ranges.

The second hypothesis, in common with other intracellular controls, has the advantage over Creighton's mechanism of potentially greater speed, and the apparent "disadvantage" of not accounting for the end-cell. In addition, it requires us to think of nervous "stimulation" of luminescence in terms of inhibition of respiration, and of the firefly as expending more energy in keeping itself dark than in luminescing.

On the whole, although a competitive mechanism is not excluded from being concerned in firefly luminescence, the probability does not seem large enough to justify pursuing the question further. Likewise, in the absence of experimental work, there seems to be little point in attempting to choose between Creighton's hypothesis and its alternative.

THE THEORY OF OSMOTIC CONTROL OF LUMINESCENCE. In 1930, Wigglesworth proposed an ingenious theory, according to which the changing needs of a cell for oxygen could be met by varying the amount of fluid in the distal end of the tracheole. This theory was

* It is generally assumed that the oxygen consumption of the luminescent reaction occurs at the same time the light is emitted. This appears to be supported by the fact that oxygen is essential for continued luminescence. However, over periods as short as those of normal flashes, it might well be that light-emission and oxygen uptake would not be simultaneous. An analogy is offered by muscle, in which the oxygen is used in the recovery or preparatory period, whereas the actual reactions involved in contraction occur anaerobically. Therefore, a comparison of oxygen uptakes during light and dark states of a tissue might not give a true measure of the extra oxygen consumption involved in the luminescent reaction.

† Some idea of the oxygen requirements of the same tissue in the luminous and non-luminous states might be obtained by comparing quiescent normal fireflies with brightly glowing "dead" specimens, or, perhaps (to eliminate the possibility of oxygen uptake by surviving tissues or by bacteria), with fireflies which had been dried and then remoistened. This would not, however, be ideal, because basal respiration would be lacking in the dead group. It may be of interest, also, to mention here my preliminary experiments on the oxygen uptake of specimens of *Photinus pyralis* poisoned with DDT. As with other insects, oxygen uptake was increased several-fold. Luminescence was also, at first, increased over the near inactivity usual in the laboratory, but consisted of irregular flashing and intermittent glowing, suggestive of nerve irritation. It was only late in the experiment, when muscular activity and oxygen uptake had both declined, that luminescence became continuous.

not originally designed for the special anatomy of the firefly, with its end-cells and anastomosing tracheoles and, moreover, it assumed intracellular penetration of the tracheoles. Several years later, however, it was applied specifically to the firefly by Maloeuf.

Wigglesworth proposed, first, that the tracheolar wall is semi-permeable. Second, he postulated that the level to which air extends in the tracheoles is determined by a balance between the osmotic pressure of the cytoplasm (which tends to pull water out of the tracheole into the cell) and the capillary attraction of the liquid in the tube (which tends to draw water out of the cell). He supported this idea by showing that hypertonic solutions injected into mosquito larvae caused movements of the air in the tracheoles. He further assumed that, if the osmotic pressure into the cell were increased during cell activity, as by the breaking-down of large substrate molecules into smaller metabolic products such as lactic acid, water would be withdrawn from the tracheole into the cell. This would allow the air to extend further in toward the cell and meet the increased need for oxygen imposed by the increased activity. Conversely, during quiescence, materials would be resynthesized in the cytoplasm, causing its osmotic pressure to fall, and water would pass from the cell into the tracheole and force the oxygen supply farther away. In agreement with expectation, Wigglesworth was able to show reversible inward movement of air, and acid formation, during anoxia. Maloeuf's contribution consisted in defining the "activity" which increases the osmotic pressure, as the luminescence of the photogenic cell, and in showing that injection of hypertonic and hypotonic solutions into the photogenic organ caused, respectively, stimulation and suppression of luminescence. Dubois, however, had found that water injection induced glowing.

The osmotic control theory has run into several difficulties. First, Wigglesworth (1938 a and b) found, in developing mosquito larvae, a number of phenomena which were not explicable by his theory in its original form. Shortly thereafter, Bult (1939) introduced a considerably altered version of the theory and, by processes too complex to elaborate here, came to the conclusion that an increase in osmotic pressure in the cell would actually bring about movement of air *away* from the cell. The "osmotic" effects he therefore attributed to swelling of cell proteins. The Bult theory could probably be adapted to the problem of photogenic control, although it is by no means clear that it is a substantial improvement over the original Wigglesworth theory. Both theories, however, suffer from the serious drawback that the observed speed of the fluid movements produced is very slow. Over distances comparable to those met in the tracheoles of the light organ, the time required is of the order of minutes, where seconds or fractions of seconds are required.

Alexander repeated Maloeuf's experiments in detail and confirmed the findings in regard to hypertonic solutions, but not concerning hypo-

tonic solutions. In some instances, moreover, he found that the direction of spread of glow in the organ did not correspond with that expected by theory. He concluded that tracheolar fluid movements are not concerned in normal flashing, although they probably are in the glow caused by injected hypertonic solutions, and in the fact that the pseudoflash has a shorter latent period than has hyperoxic glow. Since we have already seen that many substances beside hypertonic solutions induce glowing, this effect cannot safely be ascribed to osmotic changes. Likewise, as will be shown shortly, the length of the latent period between stimulus and light-production can be explained adequately by a valve mechanism which opens in low oxygen tensions and closes in high. Another explanation of the effect of fluid in the tracheoles will also be presented.

THE MECHANICAL VALVE THEORY OF END-CELL CONTROL OF OXYGEN. By far the majority of theories concerning the end-cell give it the role of a regulator of oxygen rather than a source or adjunct of luminescence. Most of these theories are in terms of the generalities which have already been discussed, as, *e.g.*, the strategic location of the end-cell, its supposed innervation, and especially its presence in flashing fireflies and absence in glowing ones.* This last point seems to me to be a particularly strong one, though, admittedly, it provides no information on how the end-cell works. The objection which is often raised that end-cells are found in non-luminous tissues, even in the firefly, does not show that they cannot operate in regulating oxygen. Schultze has pointed out that it is not the exclusive possession of end-cells, but their strikingly high concentration, which distinguishes the photogenic layer from other tissues.

Dahlgren and most other writers on end-cell physiology have assumed that the tracheoles are at least partly air-filled. As we have seen, the evidence is by no means unequivocal in this respect, but unless it is assumed that an actual current of liquid is circulated through the tracheoles, we shall have to postulate the presence of gas distal to the end-cell, as well as proximal to it, if the end-cell is to operate as a valve. This is because the speed of diffusion of oxygen through tissue is not greatly different from that through plain water (Krogh), so that, if the tracheoles were water-filled, the end-cell would offer relatively little hindrance to aqueous diffusion.

Though a number of investigators have thought of the end-cell in frankly physical terms, Dahlgren is almost alone in describing structures which might make possible the valvular action. As already mentioned, he found, in osmic acid preparations, that the tracheal twig narrows within the end-cell, loses its spiral thickenings, and shows an annular "dark-staining sheath", "specialized body", "cylindrical organ" or

* Maloeuf has made much of his claim that Bongardit found end-cells in the larva of *Phosphorus hemipterus*, an animal which, according to Maloeuf, "displays no such brisk flashing rhythm." I would not regard this as conclusive in any case, in view of the contradictions in Bongardit's paper but, actually, Bongardit (p. 24) says that end-cells are absent.

"rounded mass" ("S" in FIGURE 11).^{*} This staining reaction, continues Dahlgren, "may point to a contractile layer of cytoplasm surrounding it, with the possibility of a valve, in addition, in its lumen. Also the larger body of cytoplasm surrounding it sometimes shows a radial structure that may point to a general contractile power."[†]

Dahlgren explains the functioning of the end-cell as follows: "It is believed by the writer that these radiating rods and the rounded mass represent muscular structures which are of two possible uses: to prevent the passage of air into the tracheole by compressing them; this would appear to be the function of the rounded mass; second, to enlarge the terminal twig and end organ and thus fill it with a new supply of air; this would seem to be a possible function of the radiating rods. It can be seen that the rapid alternation of the two actions would result in the forcing of a sudden jet of air bearing free oxygen into the light cell mass, and would account for the power of flashing exhibited by the organ." Dahlgren suggests, in addition, that two sorts of nerves will be required, one for the rounded body and one for the fibrillar cytoplasm. His hypothesis was used uncritically by Creighton, Snell, and Alexander in their physiological work. Alexander, in fact, interprets most of his results on the basis of end-cells able to cause a "sudden burst of oxygen directly into the photogenic cells".

It seems to me that Dahlgren's mechanism, in the form stated, is equivalent to pinching a tapering tube open at both ends, and expecting the jet of air to move toward the smaller end (that is, into the tracheoles). Matters would be even worse if the small end of the tube were partly or entirely closed, as it might be if the tracheole contained some water or terminated intracellularly. In order to enable Dahlgren's end-cell to actually produce a "jet" of air, several additional mechanical properties must be postulated for it, and a number of new assumptions introduced. Since these requirements apply to any mechanical end-cell valve theory, it is worth while to enumerate them, as follows.

'a) The "rounded mass" must lie proximal to the region of the end-cell lumen which is enlarged by contraction of the fibrillar protoplasm. This is necessary in order that the contraction of the rounded body can shut one end of the tube through the end-cell, so as to allow pressure to build up distally.[‡]

'b) The passage through the end-cell must have an elastic wall, in order to provide a means of applying pressure to the air which is drawn

^{*} Dahlgren seems to be somewhat vague about the exact location of this body, since in his Figure 11 (p. 111) he shows it as indicated in the lower of the two end-cells pictured in my Figure 11, whereas in another version (his Figure 20, p. 345), diagrammed as the upper of the two end-cells in my Figure 11, it does not seem to appear.

[†] I, too, have seen a striated appearance in the end-cell cytoplasm in some preparations (Buck, 1940), but the structures are not very sharp. They, and indeed the whole end-cell, are very small.

[‡] Dr. H. Sneath has pointed out to me that the internal surface of the end-cell lumen must be kept dry, since otherwise a meniscus would form at the point of constriction, and resist, with relatively enormous force, the reopening of the tubule.

into the end-cell when the passage is enlarged by contraction of the fibrillar cytoplasm.

(c) The periphery of the end-cell must be rigid. This will be necessary in order to give the "fibers" something to anchor to as they pull on the wall of the lumen of the end-cell. As already mentioned, the periphery of the end-cell is notably lacking in any suggestion of such a compact structure.

d) By analogy with vertebrate sphincters, the "rounded body" ought to be composed of two layers of contractile elements at right angles to one another, one for enlarging its opening and one for constriction.*

(e) In the relaxed state of the fibrillar protoplasm, the part of the end-cell lumen which it controls must still be patent.

With these new specifications, we are in a position to revise Dahlgren's account of the operation of the end-cell. This will now require four successive stages, as follows.

First: Contraction of the fibrillar cytoplasm to enlarge the lumen of the end-cell and draw air in from the terminal tracheal twig.

Second: Contraction of the rounded mass to close off the end-cell lumen proximal to the point enlarged by the contraction of the fibrillar protoplasm.

Third: Relaxation of the fibrillar cytoplasm, so that the elastic recoil of the wall of the end-cell lumen applies pressure to the contained air and forces some of it distally into the tracheoles.

Fourth: Relaxation of the rounded mass, opening its lumen and releasing the pressure on the air in the tracheoles. Since the entire passage through the end-cell is now open, diffusion can replenish the oxygen in the tracheolar air.

Since the anatomical and operational features just outlined are so largely speculative, it is desirable to ascertain how well they meet criticisms which have been made of the concept of a mechanical end-cell valve, and how well they explain various known facts about the control mechanism.

Maloeuf raised several objections, based on his understanding that Dahlgren's theory provided for *stopping* the flash by contraction of the end-cell. This is another of the points lacking in Dahlgren's exposition. According to the revised mechanism outlined above, the decay of luminescence would be caused simply by the decrease in pO_2 due to respiration and luminescence, to a level insufficient to support luminescence. The flash is actually *started*, rather than stopped, by end-cell contraction.

* If such intricacy seems difficult to visualize in a structure as small as an end-cell, we should recall the complexity of some of the small Protozoa, to say nothing of that of an individual cilium.

Another obvious question to be asked about the mechanical end-cell control theory is whether the amount of air which could be forced into the photogenic tissue in a single "jet" would be sufficient to support the necessary luminescence. We are seriously hampered here by not knowing what that requirement is, but, if the demands of light-production are anything like those of ordinary tissue respiration, it seems unlikely that the mere contraction of the sphincter-like rounded mass (resulting in the inward movement, by a distance of a micron or two at most, of a fraction of a cubic micron of air) would be adequate. The same objection would probably apply to any theory involving simple constriction at some point in the lumen of the end-cell, even allowing for the possibility that the end-cell at the other end of the tracheole might provide a similar amount of air simultaneously. Under such circumstances, it seems that a preliminary enlargement of the end-cell passage, such as Dahlgren postulated, would be necessary. On the other hand, if the oxygen requirement of luminescence were very low, it might be possible to simplify the contractile mechanism to the extent of eliminating the expansion of the tracheolar lumen caused by contraction of the fibrillar protoplasm. The mechanism would then consist simply of two sphincters in tandem on the end-cell lumen, contracting and relaxing with the usual peristaltic rhythm.

Dahlgren describes the tracheoles as intercellular, and as connecting two end-cells of contiguous cylinders (anastomosis), but he does not consider specifically whether the "jet" is to be a *flow* of air through the open tube or a raising of the air pressure inside the tracheole. The former would result if the end-cell at one end of the tracheole were relaxed while the other was contracted; the latter, if both end-cells contracted at once. The former, or "flow" hypothesis, would have the following consequences:

(a) It might result in a more efficient renewal of the tracheolar oxygen, since air low in oxygen would be exhausted into a large space (the twig) with each pulse, where diffusion could raise its oxygen content quickly.

(b) It would require a high order of nervous coordination (alternate firing of the members of end-cell or cylinder pairs).

(c) Luminescence should spread from one end-cell toward its partner.

The "pressure" hypothesis would have the following consequences:

(a) It could operate with simultaneous firing of end-cells.

(b) Action would probably be faster than with a "flow" mechanism, since, in the latter, each flash would have to progress across the photogenic cell.

(c) It might result in a higher momentary oxygen tension in the tracheole than could be obtained by flow alone.

(d) Increase in oxygen tension would be simultaneous along the whole tracheole.

It seems to me that the points enumerated illustrate certain real advantages of a pressure mechanism. It has been pointed out previously that the final step in any oxygen control mechanism must be the passage of oxygen into the photogenic cytoplasm, by diffusion. Since the rate of diffusion cannot be changed, the problem of bringing about very rapid control becomes one of making diffusion distances as short as possible. Such a requirement is obviously not met by any arrangement whereby oxygen reaches the surface of the photogenic cell by diffusing down the tracheole. Nor is it met by a flow mechanism, because time is lost in raising the oxygen tension at successive points along the tracheole. With a pressure mechanism, however, most of the necessary oxygen can already be present in the tracheole, though ineffective in causing luminescence, due to its partial pressure being sub-threshold or because all is being used in respiration (see discussion of competition hypothesis). The pressure increase, therefore, results in a simultaneous rise in oxygen tension at all points in the tracheole, at a rate limited only by the speed of contraction of the end-cell. The rate of build-up of the flash is, then, determined by the speed of gaseous diffusion over a maximum distance of the radius of the tracheole, plus liquid diffusion in the photogenic cytoplasm. In the same way, the decay phase, or shutting-off of the flash, can occur as rapidly as the end-cell can relax,* because the oxygen tension falls simultaneously to a sub-threshold value at every point in the tracheole.

Another, if less crucial, advantage of the pressure hypothesis is that it would also apply if an inner section of the tracheole were blocked or restricted by liquid or tissue. The only effect of liquid in part of the tracheole would be to reduce the intensity of the flash by impeding oxygen access to part of the photogenic cytoplasm. In fact, such fluid might even be assigned a possible function in so varying flash intensity, thus taking care of those reports which describe fluid in the tracheoles. Moreover, since fluid would not affect the duration of the flash, one explanation is provided for Snell's observation that, although the intensity of normal flashes is highly variable, the duration is astonishingly constant. It is thus one of the advantages of the mechanical end-cell theory, in the revised form presented here, that it would operate whether the tracheoles are extracellular or intracellular, or whether they do or do not anastomose (provided that, if they do, the two end-cells of a tracheole contract simultaneously).

This whole superstructure is so complex and highly theoretical that it

* Since relaxation decreases pO_2 in the tracheole below the threshold for luminescence, decay is determined only by depletion of the oxygen actually in the cytoplasm. According to the revised version of the mechanical end-cell theory, the "contraction", or action which forces air into the tracheole would actually be a relaxation of the fibrillar cytoplasm, the force being provided by elastic recoil of the wall of the end-cell lumen. The "relaxation" would be the relaxation of the "rounded body."

may seem incongruous to speak of considering the "evidence" for one or the other hypothesis. Nevertheless, it must be kept in mind that the control of luminescence in the firefly, no matter how it is brought about, is, after all, a wonderfully precise accomplishment. It would thus be surprising, indeed, if its mechanism were not intricate, both anatomically and physiologically. At any rate, the observations of Lund, Alexander, and others, that luminescence spreads out from the cylinder wall, might be thought to favor the "flow" hypothesis. However, it must be remembered that all such observations were of glows rather than flashes and, as we have seen, the glow is most reasonably explained as due merely to diffusion through an inactivated control mechanism. In terms of the present theory, this "inactivation" would mean the relaxation (opening) of the rounded mass, without any activity of the fibrillar cytoplasm.

Further important evidence on the mechanical theory of end-cell action can be derived from considering whether it can also explain the phenomena discussed in connection with glowing. Alexander strongly emphasized the distinction between glow control and flash control, and apparently thought of the two as being quite separate, although most of his conclusions on flash control were derived from observations of glows. The points which particularly seem to indicate a dual control are the following:

(a) Voluntary flashing is abolished in the dead or decapitated animal, whereas glowing is often brilliant.

(b) Normal or electrically stimulated flashes usually occur independently of glowing.

(c) Voluntary flashing is abolished during glows produced by anesthetics or by high or low oxygen. During the latter, at least, flashing cannot even be elicited by electrical stimulation.

(d) No glow approaches in intensity the maximum reached in the flash.

(e) Under some circumstances, flashes can occur superimposed on a glow (early stages of high oxygen effect—Alexander; early stages of anesthesia or water injection—Buck).

(f) The decay portion of the pseudoflash time-intensity curve is far more gradual than that of the normal flash (Snell, Alexander).

All these observations can be explained as follows, on the basis of a valvular end-cell which has the properties which I have postulated:

(a) In the normal non-luminescent condition, the rounded body is in a state of tonic contraction such that its lumen admits only enough air to supply respiration, and the fibrillar protoplasm is relaxed, so that the part of the lumen it controls is minimal.

(b) In response to voluntary or to applied electrical stimulus, there occurs the four-stage contraction-relaxation sequence postulated as producing the air "jet." At the end of the resultant flash, the rounded body returns to its original state, with an opening insufficient to permit glow. (Flash without glow.)

(c) Anesthetics or death relax the rounded body enough so that sufficient air diffuses through to exceed the oxygen threshold for glowing. Or, alternatively, these factors depress respiration, so that oxygen becomes available for photogeny. They also inactivate the fibrillar cytoplasm, which is already relaxed (open), so that no flash is possible. (Glow without flash.)

(d) The assumption that, in ordinary air at one atmosphere pressure, glow intensity is determined only by the aperture through the rounded body, would explain why the glow (diffusion-controlled) is not as intense as the flash (pressure-controlled).

(e) A slight stimulation, preceding anesthetic or toxic effect, is a well-known pharmacological phenomenon. A slight stimulation of the fibrillar protoplasm of the end-cell, at the same time that the rounded body reached its relaxed phase, would well explain flashes superimposed on glows.*

(f) The slow decay of the pseudoflash, as compared with that of the normal flash, can be explained by a slowed-down response of the rounded body as it recovers from anoxia and resumes its tonic state of partial contraction.

In summary, the facts and assumptions presented in the last few pages suffice to explain many of the known facts about light-emission in the firefly, on the basis of mechanical control of oxygen by the end-cell. There is, however, no strong evidence that the concept is more than a hypothesis, except the correlation between the possession of end-cells and the ability to flash. Moreover, there is no proof that oxygen limitation operates directly to control luminescence. On the other hand, there exists also the hypothesis of direct nerve action, which explains known phenomena as satisfactorily as the end-cell theory. This hypothesis is supported by the fact that intensity of luminescence varies to some extent with intensity of stimulation in forms without end-cells, and by a variety of other suggestive but not "air-tight" experiments, particularly those of Bellesme, Steinach, and Snell. An obvious question is whether there is any possibility of reconciling the two ideas, so that the presence of end-cells is explicable while the apparent physiological advantages of direct nerve action are retained.

* As an additional refinement, it could be assumed that the rounded body may also contract (close) initially in response to a brief stimulation by anesthetics. This would explain Gerretsen's observation of an initial transitory suppression of luminescence by anesthetics, which Maloeuf used as an argument against the mechanical control of flashing.

An ostensible reconciliation was achieved in Brücke's (1881) suggestion that oxygen exercises control indirectly, no luminescence occurring when the direct nerves to the photogenic tissue are kept anaerobic. There is little anatomical support for this idea, nor is it easy to reconcile with, *e.g.*, the hypoxic glow. Furthermore, it apparently requires a second set of "indirect" nerves for regulating the oxygen (*e.g.*, *via* end-cells). However, the possibility of reconciling the two major hypotheses of control is so alluring that it is to be hoped that some further evidence will be adduced which points to an "indirect" function of the end-cell.

On the whole, the strongest argument against direct control of luminescence by oxygen is the fact that no other similar mechanism is known. It is true that rapid metabolic responses are, as yet, very imperfectly understood, but in the few instances which have been at least partially worked out (nerve, muscle, activation of dormant respiration), all the evidence points to enzymatic activity as the physiological "trigger" which sets off the reaction. Such enzymatic activity, in other words, appears to be the usual method of bringing about metabolic changes which are sudden and complete. There is no point in attempting to propose any specific mechanism in regard to control of luminescence, while the analogous control of muscular contraction is still unclear after an immense amount of good experimental work. However, it can be anticipated that the study of enzymatic inhibition and activation in the luminescent system will prove to be a fruitful field in future research on the control problem.

SUMMARY

In spite of the many morphological and physiological data which concern luminescence in the firefly, there seem to be surprisingly few unequivocal major conclusions which can be drawn. This is due partly to the many differences which exist between various species of fireflies and which often make generalizations impossible, and partly to the fact that our knowledge is seriously deficient in many essential points. Among the generalizations which can be made are the following:

1. The luminous organs of fireflies vary greatly in size, shape and position, and with sex and developmental stage.
2. Firefly light organs can be divided into six histological types: (1) those with no specific tracheal supply; (2) those in which the tracheae show tree-like branching and in which a "reflector" layer internal to the actual photogenic tissue is lacking; (3) those like Type 2 except for having a "reflector" tissue; (4) those in which the tracheae run through the reflector layer, branch out in the interface between reflector and photogenic layers, and terminate in "tracheal end-cells" from which

minute tracheal capillaries or tracheoles run into the photogenic tissue; (5) those like Type 4 except that the tracheae branch within the photogenic layer before terminating in end-cells and tracheoles; (6) those in which the tracheae run vertically through the photogenic layer in tissue rods called "cylinders", which contain the tracheal twigs and usually end-cells, and from which the tracheoles pass into the photogenic tissue. This sixth type occurs in most of the common adult American fireflies, while the third type is characteristic of firefly larvae.

3. The reflector layer has been shown to differ chemically and morphologically from the photogenic layer. No clear-cut evidence has been adduced as to the function or functions of the reflector layer, although numerous writers have postulated metabolic connections between it and the photogenic layer.

4. The location and morphology of the tracheal end-cell, and the fact that it is the chief site of reduction of inspired osmium tetroxide vapor, have engendered many suggestions that it functions in controlling the oxygen used in luminescence. However, tracheal end-cells, of which there appear to be at least two types, show no conclusive morphological evidence of being able to function in the way postulated.

5. Microscopic observation has shown that the light originates in the photogenic cells. The granules which are a characteristic feature of these cells are almost universally regarded as being the source of light, but no morphological evidence exists which confirms this assumption, nor the view, held by some investigators, that the granules are symbiotic luminous bacteria.

6. The anatomical course of firefly tracheoles strongly suggests that they function in conducting air to the photogenic tissue, and there is strong inferential evidence of the normal presence of air in the tracheoles, although this has not been demonstrated directly. Great variation exists in different organs, but in the most complex type the majority of evidence indicates that the tracheoles pursue an intercellular, rather than intracellular, course and anastomose directly with tracheoles from adjacent cylinders.

7. Nerves to photogenic cells or end-cells, or both, have been described. Experimental evidence indicates strongly that the nervous system, while not necessary for the production of light, does play an essential role in the control of luminescence in the normal living firefly. No conclusion can yet be drawn as to whether the nervous control is exerted directly upon the photogenic tissue, or secondarily (by way of the oxygen supply, for example). However, there is considerable evidence suggestive of the former.

8. By silver nitrate impregnation, a structure can be demonstrated which resembles an ultra-tracheolar network of tubules connecting neighboring tracheoles.

9. The normal types of light-emission in various fireflies fall into four increasingly complex classes: (a) the continuous glow; (b) the intermittent glow; (c) the pulsation; (d) the flash (of which there are several types). If various kinds of fireflies are arranged in order of increasing complexity of the air-supply to the photogenic organ, that order corresponds well to the above sequence of complexity of light-emission.

10. In the more complex types of firefly luminous organ, there are many thousands of photogenic units, each of which is active for a few hundredths or perhaps thousandths of a second.

11. Several hypothetical mechanisms for intracellular control of luminescence have been proposed.

12. It is probable that neither water, luciferin, nor luciferase is a limited reactant in the normal control of luminescence in the firefly.

13. It is certain that oxygen is essential for continued luminescence, and possible that it is the regulated reactant in the control of light-production in the firefly.

14. The spiracles have no causal connection with the control of normal flashing, or of glows caused by various experimental treatments. It is likely, however, that spiracular opening is prerequisite to long-continued luminescence.

15. The "internal control" of luminescence responds in a graded, rather than in an all-or-none fashion, and over-compensates in "opening" in response to lowered oxygen.

16. The simplest assumption in regard to the normal states of both spiracles and internal control is that they are in tonic contraction (closed). Partial anoxia, anesthesia and similar treatments would, thus, produce a relaxed (open) condition, permitting luminescence.

17. There is no anatomical evidence specifically identifying the internal control with the tracheal end-cell, although in the more complex types of firefly organ, where luminescence can be controlled rapidly, there is strong indirect evidence that the tracheal end-cell is concerned in control. However, firefly larvae, which lack end-cells, exhibit internal control of glowing similar to that in adults, indicating that end-cells need not be concerned with glow control even if they are concerned in flash control.

18. Some theoretical implications of the hypothesis that light-production is controlled by competition for oxygen between the luminescent reaction and respiration are discussed.

19. The hypothesis that access of oxygen to the luminescent tissue is controlled by changes in the fluid content of the tracheoles, is discussed and found to be deficient in some respects.

20. The hypothesis that oxygen access is limited by a valvular action of the tracheal end-cells is discussed in detail and found to accord well with what is actually known at the moment about control of flashing in the firefly. Like others, however, the end-cell hypothesis requires the use of a number of assumptions which, so far, it has been impossible to test experimentally.

21. Arguments are presented for regarding enzymatic mechanisms as better fitted than oxygen regulation for the rapid and complete control of luminescence.

Almost all of the work reported in this paper was concerned, either directly or indirectly, with the general question of what mechanisms, external to the photogenic cell, affect its luminescence. In considering the possible trends of future work in this field, it seems clear that important new departures cannot be expected until definite solutions are obtained to a number of long-recognized basic problems. Most of these problems center around the question of whether the photogenic cell is stimulated by direct nerve action, presumably in an environment always adequate in oxygen, or whether the stimulus which sets off luminescence is the passage of oxygen into the cell.

As already related, it has so far been impossible to devise a physiological experiment by which the respective effects of nerve action and of oxygen can be separated with certainty. One of the most promising future experiments is the simultaneous recording of light-emission and of the spontaneous action potentials of the central nervous system. Yet, even if a good correlation were found between the potential pattern and luminosity, direct nervous action would not be proved. It could be argued that the nerves had been stimulating some indirect agency, such as the end-cells, which, in turn, brought about direct stimulation of the photogenic cell by oxygen. Another significant type of approach involves a careful physiological study of two species of lampyrid fireflies, one of which has end-cells and the other has not.

The most direct lines of attack, at the moment, seem anatomical. Conclusive establishment of the ultimate terminations of the nerves to the photogenic organ, if carried out both in fireflies with end-cells and in those without, might go far in resolving the present impasse. Conversely, any cytological evidence bearing on the structure and activity of

the end-cells and tracheoles in the living photogenic organ, both in the luminescent and non-luminescent state, could hardly fail to be significant. Likewise, evidence that end-cells can regulate oxygen rapidly in non-luminous insects, would be of great value. Furthermore, it would be of at least theoretical interest to calculate the potentialities of gaseous diffusion, flow, and pressure, in supplying air to the photogenic tissue through conducting systems of the known anatomical dimensions.

Further important information on the possibility of direct nerve action might be obtained by carefully controlled experiments on electrical stimulation of the nerve cord, with special reference to the relation between length or strength of stimulus and total resulting luminescence, total luminescence in relation to oxygen tension, and total luminescence in relation to length of preceding anaerobiosis. Oxygen tensions used should be kept within the range of 100 to 300 mm., in which many physiological reactions remain constant, and particular attention should be paid to fireflies lacking end-cells.

Finally, the simplest and most important desideratum in future work on fireflies is improvement in the design of experiment. It cannot fail to impress anyone familiar with the literature, that an enormous amount of work has been weakened or negated by failure to observe such elementary methodological precautions as the use of adequate controls, frequent repetitions, the variation of only one experimental factor at a time, and appreciation of the range and frequency of the normal and experimental variation of the material.

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FIGURES 1-41**Key**

C, cylinder; CM, cell membrane; CU, cuticle; DZ, differentiated zone of photogenic cytoplasm; EC, tracheal end-cell; EN, end-cell nucleus; EP, end-cell process; FB, fat body; M, muscle; N, nerve; NE, epithelial cell nucleus; O, region where osmium has reduced in the photogenic cytoplasm; P, photogenic layer or tissue; PC, photogenic cell; PN, nucleus of photogenic cell; R, reflector layer; RC, reflector cell; S, "rounded body"—a possible sphincter; T, trachea; TE, tracheole; TW, tracheal twig.

Unless otherwise specified in the figure legends, the preparations used for the photomicrographs are 10-micron sections, stained with Delafield's hematoxylin and eosin. Fixation was with Bouin's fluid, except for the preparation for **FIGURE 32**, for which hot Bouin's was used. Detailed techniques will be published elsewhere.

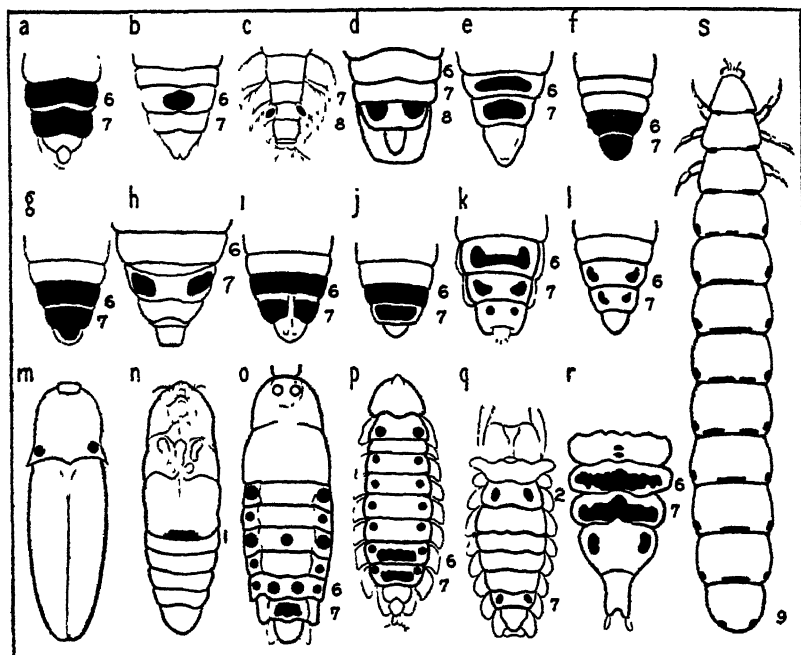


FIGURE 1. Outline diagrams showing positions and sizes of photogenic organs in representative fireflies. Photogenic organs indicated in solid black. Numbers refer to abdominal segments. Ventral views, unless otherwise noted. Sizes are variable and not shown to scale, but normal size of *Phengodes* (s) is about 40 mm., *Pyrophorus* 25 mm., entire "average" firefly 10-15 mm. a, *Photinus scintillans*, male; b, *Photinus scintillans*, female; c, *Photuris pennsylvanica*, larva; d, *Diphotus montanus*, male; e, *Photuris pennsylvanica*, female; f, *Luciola chinensis*, male; g, *Luciola cruciata*, male; h, *Luciola lusitanica*, female; i, *Luciola* sp., male; j, *Luciola lateralis*, male; k, *Pyrocoelia rufa*, female; l, *Lecontea lucifera*, female; m, *Pyrophorus noctilucus*, dorsal; n, *Pyrophorus noctilucus*, ventral; o, *Lamprohiza (Lamprohiza) splendidula*, female (the number of lateral organs is quite variable, according to Vogel, 1922); p, *Phaustis mulsanti*, female (the number of lateral organs is variable); q, *Phaustis Delarouzei*, nymph, dorsal; r, *Lamprohiza noctiluca*, female; s, *Phengodes* sp., dorsal. Figures a, b, c, and e after Hess (1920). Figures f, g, i, j, k, and l, after Okada (1935 b). Figures h, h, p, and q, after Bugnion (1929). Figure o, from various sources. Figure r, after Bongardt (1903). Figures d and s, original. Figures m and n from Seaman (1891).

FIGURES 2-7 (see opposite page).

FIGURE 2. Two end-cells from male of *Lamprohiza splendidula*. Osmic acid vapor impregnation; x 650. After Bongardt (1903).

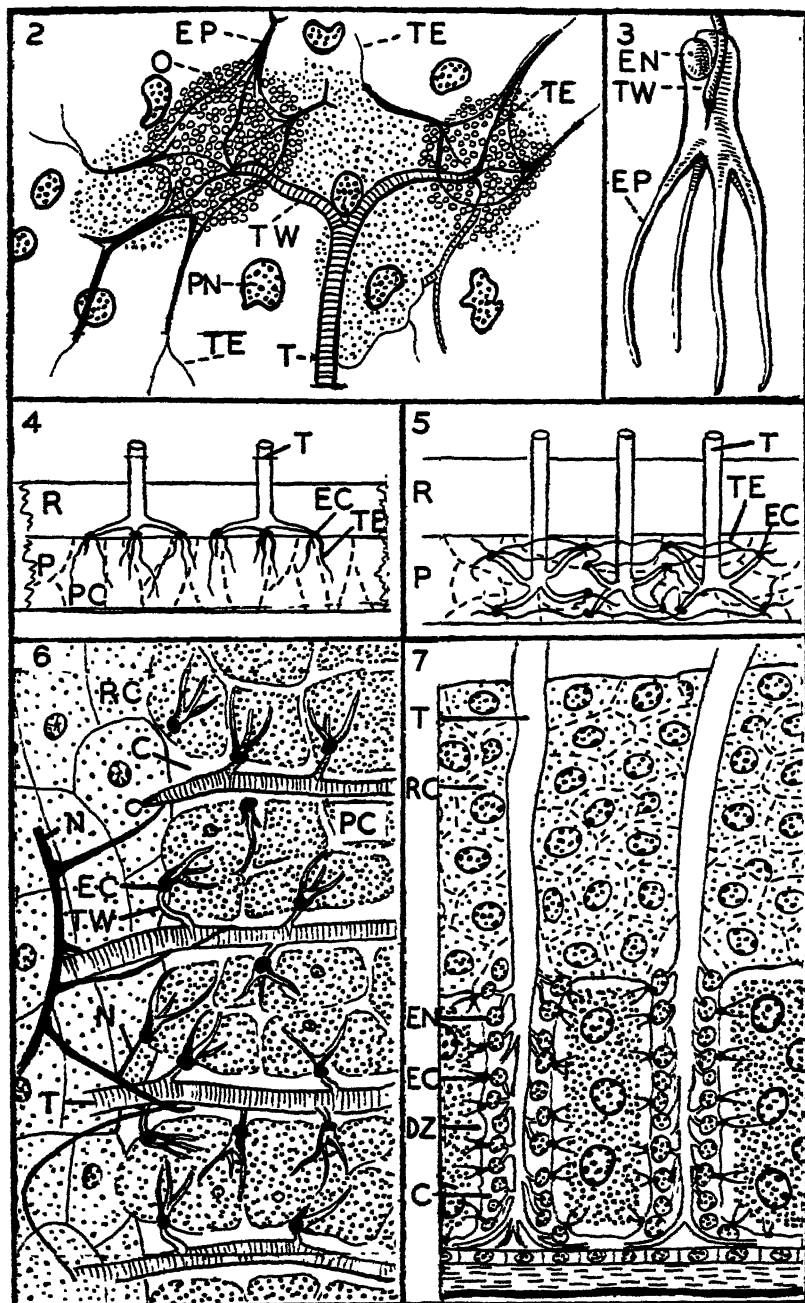
FIGURE 3. Tracheal end-cell from *Photinus marginellatus*. Osmic acid impregnation; x 1700. After Geipel (1915).

FIGURE 4. Diagram of Type 4 arrangement of tracheae. End-cells at boundary between reflector and photogenic layers, with tracheoles extending into latter. After Dahlgren (1917).

FIGURE 5. Diagram of Type 5 arrangement of tracheae. Trachea branching into interior of photogenic layer and there terminating in end-cells. After Dahlgren (1917).

FIGURE 6. Arrangement of end-cells and nerves in the light organ of *Photinus marginellatus*. After Geipel (1915).

FIGURE 7. Cross section of the light organ of adult *Photuris pennsylvanica*. After Hess (1922). Contrast with FIGURE 11.



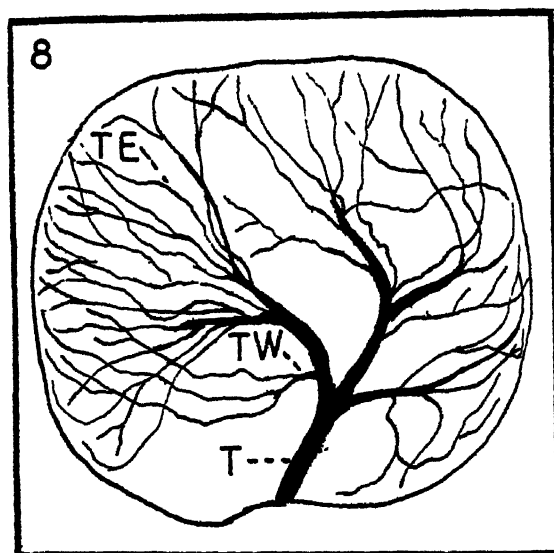


FIGURE 8. Larval organ of *Phosphaenus hemipterus* from caustic potash preparation to show tree-like branching of tracheae. After Bongardt (1903).

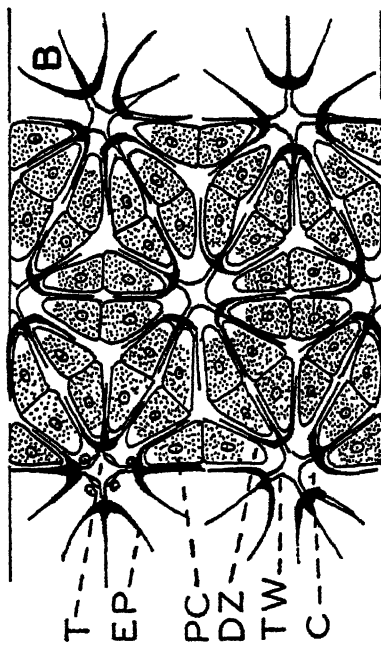
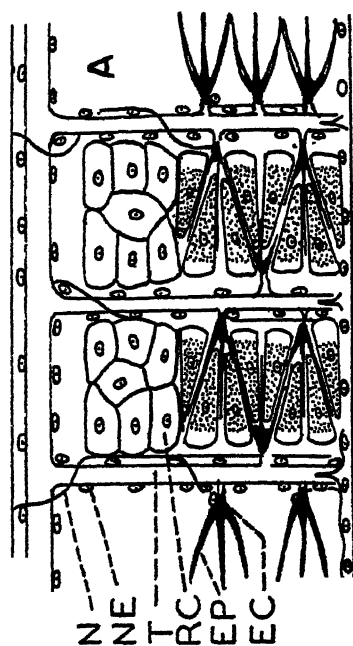


FIGURE 10. Diagram of cross (above) and horizontal (below) sections of the light organ of *Photinus* sp. (Presumably Korean). This is a variant of the Type 6 system of tracheal branching. After Okada (1935 b).

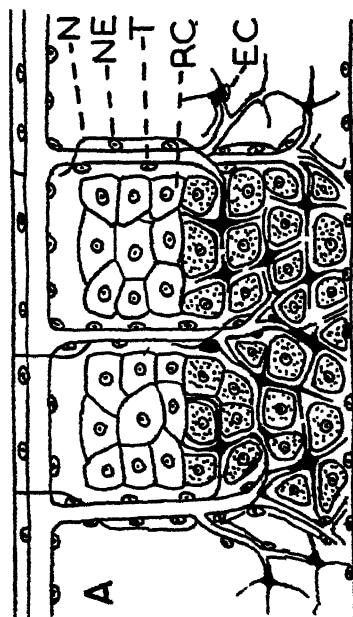


FIGURE 9. Diagram of cross (above) and horizontal (below) sections of the light organ of *Lucania crataea*. This is a variant of the Type 6 system of tracheal branching. After Okada (1935 b).

FIGURES 11-14 (see opposite page).

FIGURE 11. Composite diagram of end-cell and cytoplasmic structure from Figures 11, 16 and 20 of Dahlgren (1917). Lower end-cell more heavily impregnated with osmic acid than upper, and showing "fibers" in its cytoplasm and a "rounded body" (s) with postulated constrictor powers. Rounded photogenic granules in upper cells of male type; rod-shaped granules in lower photogenic cell of female type.

FIGURE 12. Cross section of light organ of *Pyrophorus*. After Dubois.

FIGURE 13. Diagram of horizontal section through light organ of *Photinus marginellus* to show "rosette" pattern of tracheolar anastomoses between cylinders. Diameters of tracheoles exaggerated. Upper two cylinders show terminal brushes of tracheal twigs, lower shows nuclei of tracheal epithelium and end-cells; approx. $\times 1000$. Partly after two figures of Townsend (1904).

FIGURE 14. Cross section of light organ of female of *Photuris pennsylvanica* from osmic acid preparation. After Lund (1911).

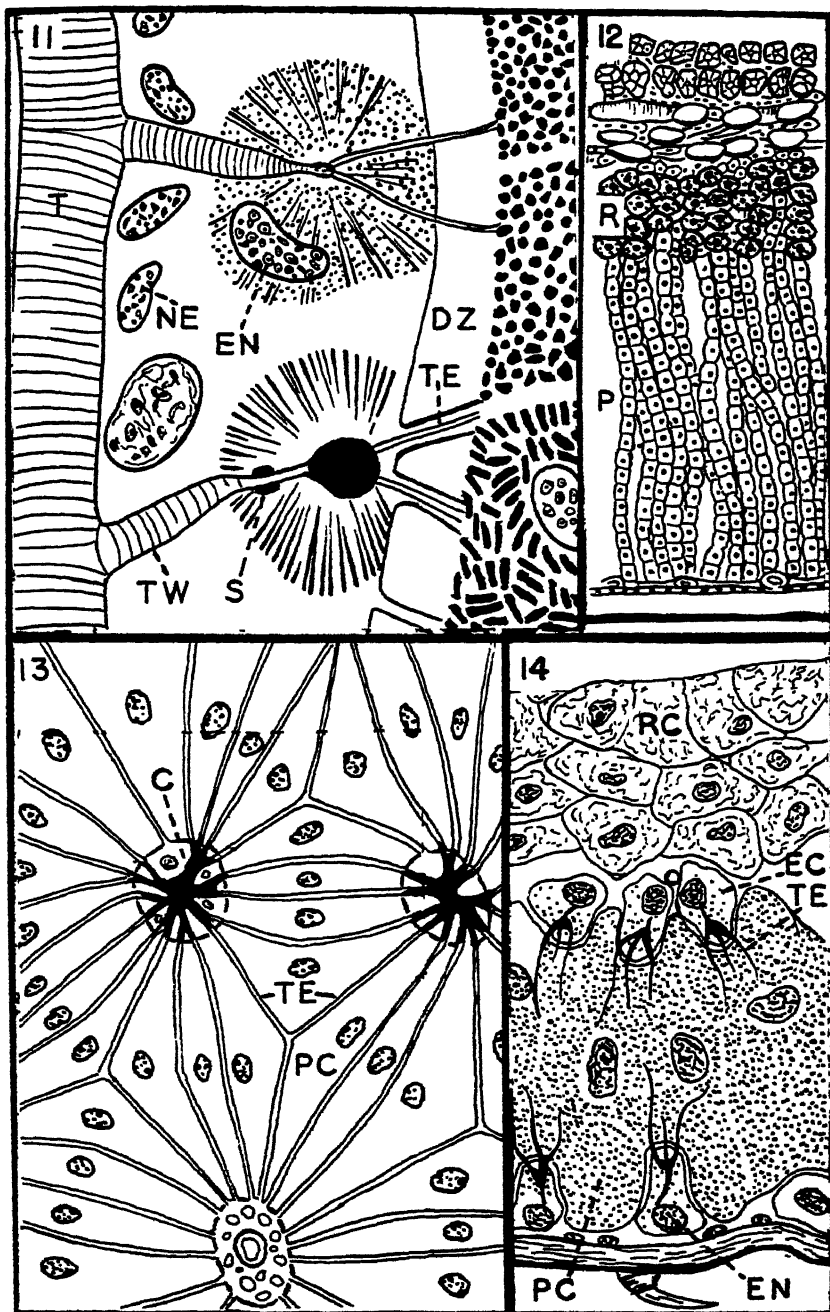


PLATE 1

FIGURE 15. *Phengodes* sp. Longitudinal section of lateral luminous organ, X180. Large oenocyte-like photogenic cells loosely aggregated in a fold of the external cuticle. The photogenic cells are apparently slightly shrunken.

FIGURE 16. *Phrixothrix* sp. Cross section of lateral luminous organ; X200. Organ is situated amid fat-bodies beneath the dorsolateral cuticle. Clear spaces between photogenic cells are small tracheal branches.

FIGURE 17. *Photuris pennsylvanica*. Cross section of luminous organ of larva; X180. The two white dots at the apex of the photogenic layer are cross-sections of tracheae. Ventral cuticle slightly retouched.

FIGURE 18. *Pyrophorus plagiophthalmus*. Cross section of abdominal organ; X250.

FIGURE 19. *Diphotos montanus*, female. Cross section of part of luminous organ, X250. The separation between photogenic and reflector layers is an artifact.



BUCK: THE LIGHT ORGAN IN FIREFLIES

20



BUCK: THE LIGHT ORGAN IN FIREFLIES

PLATE 2

FIGURE 20. *Photuris jamaicensis*, female. Cross section through entire abdomen, to show location of light organ on the ventral surface: X25. Note the high concentration of large tracheae on the internal surface of the reflector layer.

FIGURE 21. *Photinus lan'hophoris catherinae*, male. Horizontal section through photogenic organ, to show the tracheal trunks arranged in triangular symmetry and the "rosettes" of photogenic cells around each: X96. The nuclei of the photogenic cells are arranged approximately equidistant from adjacent cylinders. The nuclei grouped close around each trachea belong mainly to tracheal end-cells, which are particularly abundant at the ventral surface of the organ—see also FIGURE 39. Compare with FIGURES 22, 29, and 36.

FIGURE 22. *Photinus euphorus*, male. Horizontal section through photogenic organ, showing "rosette" arrangement of photogenic cells around the cylinders: X540. This view also shows end-cells, tracheoles, cylinders, and the differentiated zone of the photogenic cytoplasm. Compare with FIGURES 21, 29, and 36.

FIGURE 23. *Photinus pyralis*, male. Cross-section through light organ. X25. This organ is characterized by large tracheal trunks, long narrow cylinders, photogenic and reflector layers of approximately the same thickness and number of cell layers, 7 or more. One or two of the most internal of the photogenic cells show a lighter cytoplasm, such as has been described for "transition cells" between the photogenic and reflector layers—page 436. Compare with FIGURES 24, 25, 26, 31, 32, and 35.

FIGURE 24. *Photinus synchronans*, male. Cross-section through light organ. X250. This organ has narrow tracheae, and photogenic and reflector layers each about 5 cells thick. Compare with FIGURES 23, 25, 26, 31, 32, and 35.

PLATE 3

FIGURE 25 *Photinus amicus* male. Cross-section through luminous organ, X250. In this species, the photogenic layer is usually thinner than the reflector, and the cylinders are often greatly flared at the ventral (and often the dorsal) surface of the photogenic layer. Compare with FIGURES 23, 24, 26, 31, 35, and especially 32.

FIGURE 26 *Photinus pallens* male. Cross-section through photogenic organ, X210. Specially stained (first method of Severinghaus) to accentuate the differentiated zone of the photogenic cells. Also shows cylinders expanded in the interior of the photogenic layer, and tracheoles. Compare with FIGURES 23, 24, 25, 31, 32, and 55.

FIGURE 27 *Photinus pallens* male. Three separated cylinders from osmic acid maceration preparation. X500. The end-cells show varying degrees of impregnation from light (in the upper part of the right-hand cylinder) to heavy (in the upper part of the left-hand cylinder). A few tracheoles show in upper left and lower right. Note the profusion of trichial twigs and end-cells.

FIGURE 28 *Photinus pyralis* male. Ventral surface view of photogenic organ which has been freshly peeled off. X500. The brushes of tracheae and twigs are especially profuse at the surface, since the cylinders usually flare there. Dark "wall" of cylinders may be the differentiated zone of the photogenic cytoplasm. Compare with FIGURES 29 and 38.

FIGURE 29 *Photinus pyralis* male. Ventral view of photogenic organ which has been peeled off and partly dried. X790. Deeper focal level than FIGURE 28. Note tracheoles running between contiguous cylinders (several anastomoses are visible). Origin of tracheoles within cylinder visible in the central cylinder and the one to the left of it. Compare with FIGURES 28 and 38.

FIGURE 30 *Photinus pallens*, male. Lateral view of terminal part of tracheal trunk of cylinder, from fresh smear. X500.



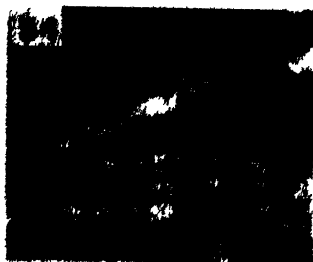
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FIGURE 1. THE LIVER OF THE FISHES



SPOROZYTES OF *SPOROZYTOXIS* IN *SPOROZYTOXIS*

PLATE 4

FIGURE 31. *Photinus evanescens montego*, male. Cross-section through photogenic organ; X920. This species has a very thin photogenic layer, with extraordinarily broad cylinders. At least 7 end-cells show on the right edge of the cylinder in the center, and only three photogenic cells (judged from the number of nuclei). Note also the sharp delimitation of end-cell cytoplasm and photogenic cytoplasm. The differentiated zone of the photogenic cytoplasm, tracheal twigs, and several tracheoles show as well. Nuclei of both end-cells and tracheal epithelial cells occur in the cylinder. Compare with FIGURES 23, 24, 25, 32, and 35.

FIGURE 32. *Photuris pennsylvanica*, male. Cross-section of photogenic organ, X620. This figure shows the end-cells with their tapering processes, but tracheoles are not visible. In the lower right, where the end-cells are cut transversely, note that each has 4 processes. Note also end-cell processes entering the photogenic cytoplasm from both surfaces of the photogenic layer. Nuclei of end-cells not distinguishable tracheal epithelial cells. Compare with FIGURES 23, 24, 25, 31, and 35.

FIGURES 33 and 34. *Photuris jamaicensis*, male. Horizontal section of the photogenic organ impregnated with silver nitrate. X2300. These figures illustrate the delicate network which runs on the interfaces between the photogenic cells and binds contiguous tracheoles together.

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RECENT STUDIES IN THE
MECHANISMS OF EMBRYONIC DEVELOPMENT

BY

E. J. BOELL, D. P. COSTELLO, S. R. DETWILER,
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J. S. NICHOLAS, S. M. ROSE,
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RECENT STUDIES IN THE MECHANISMS OF EMBRYONIC DEVELOPMENT*

Consulting Editor: ROBERTS RUGH

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* This series of papers is the result of a Conference on The Mechanics of Development, held by the Section of Biology of The New York Academy of Sciences on January 10 and 11, 1947, with Roberts Rugh of New York University as Organizing Chairman. Publication made possible through a contribution from the General Funds of The New York Academy of Sciences.

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OOPLASMIC SEGREGATION IN RELATION TO DIFFERENTIATION

By DONALD PAUL COSTELLO

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OF the many processes which take place during the maturation, fertilization, and early cleavage of invertebrate eggs, it is apparent that some are more significant than others in causally contributing to differentiation. The fact of parthenogenesis indicates that the contributions of the spermatozoon to development, particularly its nuclear contribution, cannot be of primary significance. The fact that certain ova, under experimental conditions, may show various degrees of differentiation without cleavage, indicates that neither the mitotic mechanism, nor cleavage, nor the cleavage pattern, nor cell boundaries are of essential importance. It is likewise clear, from experimental evidence as well as by genetic assumption, that the differential competence of the different blastomeres of the early embryo cannot be accounted for on the basis of nuclear, chromosomal, or genic differences between the blastomeres. We are, thus, led to the conclusion that the factors necessary for differentiation are those producing the localization of the cytoplasmic areas of specific potency—or, if one wishes to use another terminology, the factors producing cytoplasmic fields and gradients. The study of such factors may be approached from the standpoint of metabolic measurements, in terms of differential metabolic rates and enzyme distribution. However, I believe that a more direct *biological* approach to the problem is afforded by the study of the process that has been termed oöplasmic segregation.

The segregation of the visible cytoplasmic elements in the eggs of marine invertebrates was early described by Wilson (1892), Conklin (1905), Lillie (1906), and others, and has been more recently studied by Spek (1930, 1934a, 1938) under the term, *bipolar differentiation*. It is the purpose of the present paper to review the recorded facts in relation to oöplasmic segregation and to make some suggestions concerning the significance of the process.

The phenomenon of visible oöplasmic segregation is particularly striking in eggs showing the so-called "determinate" type of cleavage, and may be initiated, in different forms, at the time of, or prior to, germinal vesicle breakdown, during polar body formation, or at fertilization or parthenogenetic activation. Invertebrate eggs of the various animal groups may be divided into different categories as regards the time of onset, and the pattern, of oöplasmic segregation. There are also different relationships between the pattern of segregation and the cleavage

pattern. Because of these various types of segregation patterns, Spek's term, bipolar differentiation, is a misnomer. Since there is an original polarity of the egg that can be traced back to the earliest stages of the oögonium, it is recognized that the simplest type of oöplasmic segregation is essentially a re-polarization of certain of the egg constituents.

Oöplasmic Segregation in the Egg of NEREIS. The bipolar pattern of oöplasmic segregation is, perhaps, best exemplified by the *Nereis* egg. Spek (1930) studied the process in the egg of *Nereis dumerillii*, using vital staining methods. The process of segregation of the oöplasmic constituents does not begin until after extrusion of the polar bodies (FIGURES 1 and 2). It is especially apparent at the four-cell stage, as figured by Spek (1934a) for *Nereis limbata*, where the animal hemisphere gives an alkaline reaction with indicator dyes and the vegetal hemisphere gives an acid one. This has been discussed in considerable detail by Costello (1945a) for the egg of *Nereis limbata*. Since the animal hemisphere is destined to give rise in development to the ectodermal quartets of micromeres, whereas the vegetal hemisphere produces the endodermal macromeres, the implication of Spek's work is that there is a causal relationship between this "bipolar segregation" of acid and alkaline "protoplasm" and the differentiation of endoderm and ectoderm. I shall return later to the discussion of this point.

In normal eggs of *Nereis*, the time of onset of visible oöplasmic segregation is shortly after the final incorporation of the sperm head into the egg. Since an accentuated aggregation of cytoplasmic components is obtained in *Nereis* eggs from which the activating spermatozoon is removed (along with the vitelline membrane) by alkaline sodium chloride (Costello, 1945b), sperm entrance is not a necessary prerequisite. Certain salt solutions (Spek, 1930, 1934b) may also induce segregation in unfertilized eggs.

Oöplasmic Segregation in the Egg of CHAETOPTERUS. In the egg of *Chaetopterus*, segregation takes place much earlier than in that of *Nereis*, and it is essentially completed by the metaphase of the first maturation division (Lillie, 1906). The ectoplasm of the ovarian egg covers the free hemisphere and ends a short distance below the equator, so that the endoplasm comes to the surface in the vegetal hemisphere. There is usually also a small ectoplasmic defect at the animal pole, where the endoplasm comes to the surface. After the egg is shed by the female *Chaetopterus* into sea water, the germinal vesicle ruptures and a series of movements of egg substance takes place. The ectoplasm flows toward the vegetal pole, covering the exposed endoplasm. The original polar defect enlarges and the maturation spindle becomes attached there. There is also a redistribution of the different types of endoplasmic granules *a*, *b*, and *c* (see Lillie, 1906, Figures 1, 2, 5, and 25). This distribution of substances corresponds, in many respects, to the future embryonic areas and is maintained from this time on. At the first cleavage, the polar



1



2

FIGURE 1 Section of fertilized egg of *Nereis limbata*, at prophase of first maturation division. Note chromosomes and nucleolus in rupturing germinal vesicle, and concentric orientation of oöplasmic inclusions as in unfertilized egg. Photomicrograph, magnification 525 \times .

FIGURE 2 Section of fertilized egg of *Nereis limbata*, at metaphase of first maturation division. The oil and yolk have been dissolved by the reagents. There has been no segregation of inclusions other than that brought about by movement of the spindle toward the animal pole. Presumably, the spindle is formed by rearrangement of the structural proteins of the protoplasm. Photomicrograph, magnification 590 \times .

bodies and ectoplasmic defect are transmitted to the *CD* blastomere, and at the third cleavage the polar defect is found on the *1d* blastomere, which gives rise to the apical tuft. A more detailed account of the be-

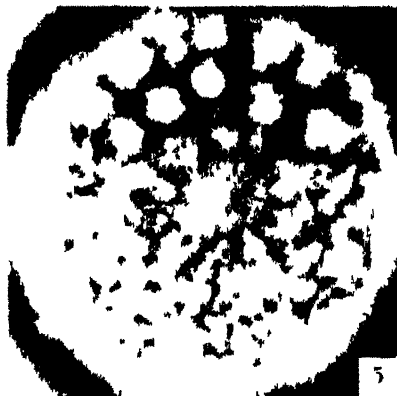


FIGURE 3 Unfertilized *Veris* eggs centrifuged 60 minutes at about 6000 times gravity, photographed 5 minutes after centrifuging. The large oil droplets mark the centripetal end. All inclusions are sedimented into strata. Magnification 135 \times .

FIGURE 4 *Veris* egg centrifuged 24 minutes in 0.73 M sucrose at 6000 times gravity, inseminated immediately, and photographed 53 minutes later. Note oil droplets marking centripetal pole. Polar area, in surface view, is at equator. Magnification 410 \times .

FIGURE 5 *Veris* egg centrifuged 10 minutes in 0.73 M sucrose at 6000 times gravity, inseminated immediately, and photographed 70 minutes later. Note oil droplets, marking centripetal pole, at top, polar area and first polar body in surface view at equator. Magnification 410 \times .

havior of the *Chaetopterus* egg during this segregation period is given by Lillie (1906) and summarized with special reference to the possible rôle of the polar defect, by Costello (1945a).

The Mechanism of Oöplasmic Segregation With these brief descriptions of two examples of the process of oöplasmic segregation, we have materials for a consideration of the mechanism involved. Spek (1930, 1934a, 1938) speaks of the process as being brought about by a "self-cataphoresis," but there is no evidence for a flow of electric current through the cell, and no evidence that such a distribution of substances is obtained when a potential difference is superimposed upon the cell from outside. Nor will the action of gravity (or magnified gravity, as in a centrifuge) in sedimenting the contained oöplasmic inclusions produce a similar pattern. For example, in oöplasmic segregation in the *Nereis* egg, oil and yolk go toward the vegetal pole, whereas in the centrifuge (FIGURE 3), oil moves centripetally, yolk centrifugally, to opposite directions, which are usually at right angles to the animal-vegetal axis (FIGURES 4 and 5). This is due to orientation of the somewhat disc-shaped egg in the centrifuge, with polar axis (short axis of the disc) at right angles to the direction of the centrifugal force.

An explanation of oöplasmic segregation was recently proposed (Costello, 1945a) in terms of the Teorell "diffusion effect." As illustrated in FIGURE 6, the Teorell system is as follows: Across a permeable bound-

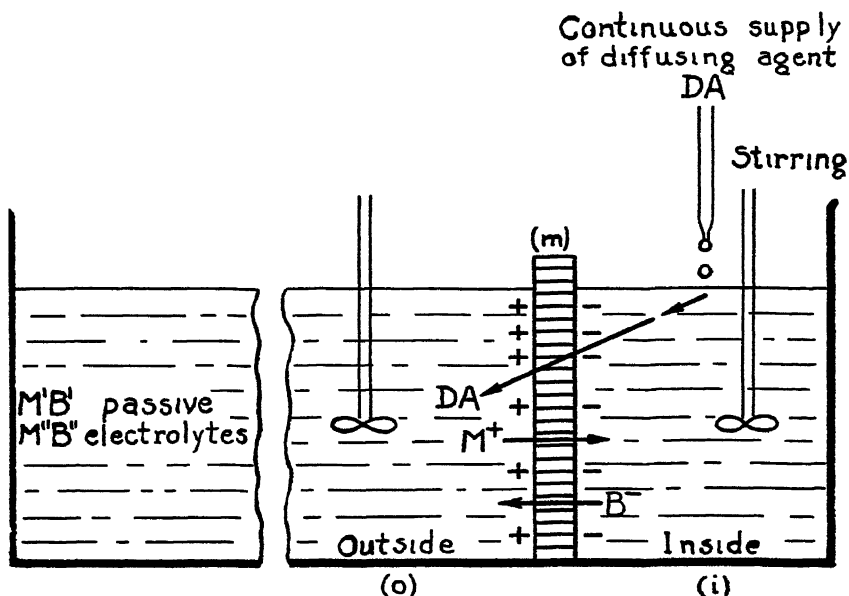


FIGURE 6 Diagram to illustrate Teorell's experimental arrangement for demonstration of the 'diffusion effect' (After TEORELL, 1937.)

any (*m*) there is assumed to be present a constant difference in concentration of either the cation D^+ or the anion A^- . The maintenance of this condition is accomplished by the continuous addition of the substance DA to the small volume (*i*) [= inside], causing DA to act as a "diffusion agent" which steadily diffuses across (*m*) into the part (*o*) [= outside]. In (*o*), a fixed, constant composition is maintained by keeping the volume large.

The continuous steady diffusion of DA was shown by Teorell (1935a, 1937) to influence the distribution of other electrolytes present, denoted by M^+B^- , M^+B^- , etc., which were not participating in any active diffusion and were therefore called "passive ions." The membrane is permeable to these passive ions. It was assumed that the D^+ ions had a higher mobility in the boundary (*m*) than the A^- ions, producing an electrical potential across the boundary. Starting with the initial state of equal concentrations of M^+ and of B^- on the two sides of the membrane (*m*), the electrical potential causes an inward migration of M^+ and an outward migration of B^- . Finally the concentration gradients become sufficiently large to balance the electrical gradient and the system approaches a steady state. Thus, the M^+ ions accumulate and B^- ions decrease in amount inside. Therefore, a diffusion of one electrolyte may produce, inside, an accumulation or impoverishment of other cations or anions, depending upon the mobilities of the ions of the diffusing agent. This effect upon ionic distribution is called the diffusion effect.

Teorell's concept of diffusion effect was later extended (1935b) to apply to any electrically charged particle, regardless of size. The diffusion potentials present in the system would thus move positively charged particles into the negative part of the diffusion potential field, and negatively charged particles in the opposite direction. It was emphasized that this "diffusion effect" upon ionic and colloidal distribution is not a cataphoretic effect, because no current is flowing and no external E.M.F. is applied. It is brought about by exchange of charged particles due to differences in mobility of the ions of the diffusing substance within the membrane.* Teorell (1935c) also devised a scheme for studying ionic distribution within a thick diffusion layer—the so-called multi-membrane arrangement.

In my 1945 paper, I proposed a biological analogue of the Teorell scheme to account for ooplasmic segregation (FIGURE 7). If there were a continuous supply of diffusing substance entering the cell across a special area of the cell membrane, such as the polar area, diffusion potentials might be set up within the egg, with part of the protoplasm (such as the protein framework) acting as a multimembrane. If electrically charged particles, regardless of their size, were subjected to the influence of these diffusion potentials, positively charged particles would be moved toward one pole of the egg, negatively charged particles toward

* The normal difference in mobility of the ions of a substance is sufficient, but this mobility difference may be accentuated by certain types of membranes (Teorell, 1937).

the other. In this earlier paper, I considered at some length the assumptions basic to the application of the Teorell scheme in such a case, and the reader is referred there for further details. I should like to point out that Harrison (1945) has recently visualized the ovum (Harrison, Text figure 12) in a manner essentially corresponding to my last figure, with a protein framework and distribution within this framework of two types of substances. Harrison distinguishes two types of polarity in the ovum: a polarity of direction (*Richtungspolarität*) and a polarity of stratification (*Schichtungspolarität*). The former is equivalent to what I have called the original polarity of the egg, the latter to what is brought about by the process of segregation.

If oöplasmic segregation is initiated when the egg enters sea water, or when some event, such as fertilization, alters the membrane permeability to permit entry of the diffusing substance, then the diffusing sub-

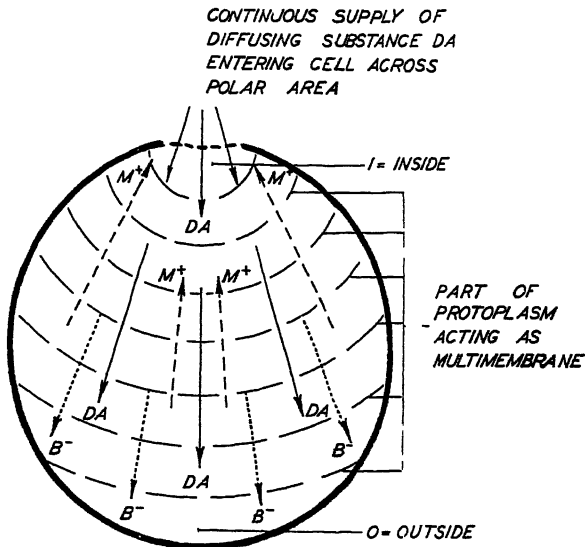


FIGURE 7. Biological analogue of Teorell scheme, employing "multimembrane" or thick diffusion layer to bring about segregation of particles M^+ , B^- within cell. (After COSTELLO, 1945a.)

stance is probably either one of the common constituents of sea water, or a constituent of the outer surface of the egg which is able to enter the egg after its permeability is altered. Spek (1930, 1934b) has induced an artificial "bipolar differentiation" in the eggs of *Nereis* and *Asterias* by means of potassium chloride. Mead (1898) induced *Chaetopterus* eggs to complete their maturation by the addition of a small quantity of potassium chloride to sea water. It is also this substance which induces *Chaetopterus* eggs to undergo differentiation without cleavage (Lillie, 1902, 1906). There is, therefore, the possibility that the diffusing substance may be potassium chloride. However, it is very important

that the diffusing substance should show a considerable difference in mobility of its ions within the "membrane" [*i.e.*, (*m*)], in order to set up a diffusion potential of any great magnitude. The mobilities of the potassium and of the chloride ion are almost the same in simple solutions. We do not know with certainty what their relative mobilities would be within the egg protoplasm.

It is a well-known fact that modification of the external medium, as

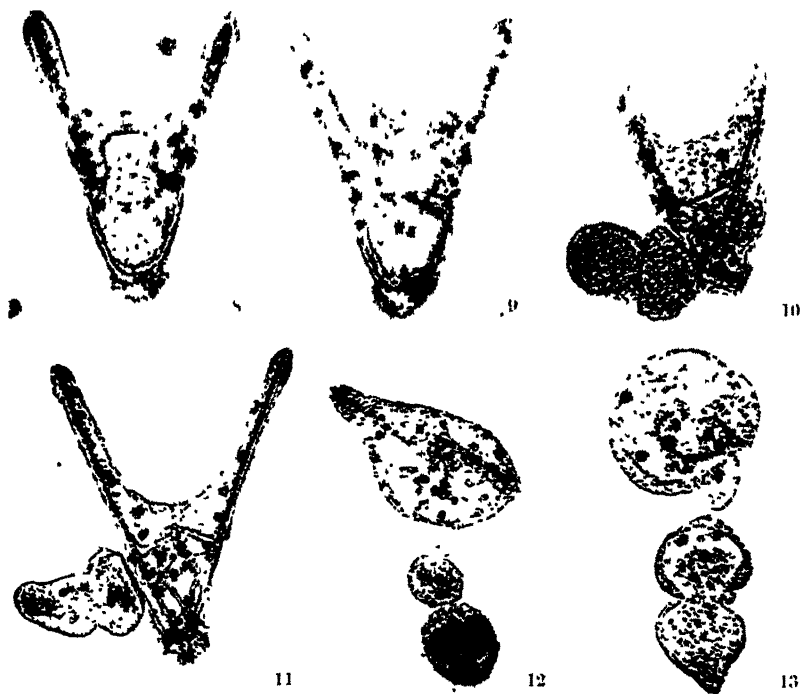


FIGURE 8. Normal, living pluteus of *Arbacia punctulata* about 40 hours old. Viewed from aboral surface, magnification 150 \times .

FIGURE 9. Same, viewed in median optical section.

FIGURE 10. Exogastrulated pluteus, 48 hours old, developed from *Arbacia* egg treated for 4 hours with mixture of 0.54 M LiCl (20 parts) and sea-water (80 parts), beginning 53 minutes after insemination (during 2-cell stage). Magnification 150 \times .

FIGURE 11. Exogastrulated pluteus, 52 hours old, same treatment as FIGURE 10. Magnification 130 \times .

FIGURE 12. Exogastrulated pluteus, 57 hours old, same treatment and magnification as FIGURE 10. Effect more extreme.

FIGURE 13. Exogastrulated pluteus, 52 hours old, same treatment and magnification as FIGURE 12. Effect still more extreme.

by the addition of a simple salt such as lithium chloride, leads to modification of development. Some examples of lithium-induced exogastrulation in the sea urchin, *Arbacia punctulata*, are shown in FIGURES 8-13.*

* Observations made by Mrs. Marjorie Hopkins Fox and the author in 1945.

It is possible that the lithium acts through an effect on the diffusion potential and oöplasmic segregation. *Arbacia* embryos from a given culture which has been subjected to a specific dosage of lithium chloride to induce exogastrulation, exhibit a remarkable variation in the degree of developmental modification. Child (1940) has exhaustively discussed this variation in susceptibility to lithium. He suggests that aggregation at the bottom of the container produces environmental conditions different from those surrounding isolated individuals. One might argue that an internal protoplasmic gradient would tend to produce just such a high degree of variability if the eggs come to rest with their polar axes oriented at any angle with respect to the bottom of the dish. That is, in some cases, the external chemical gradient effect would be added to the original polar gradient; in other cases, the two would act in opposite directions; and in still other cases, at all possible angles to each other.

Since the early experiments of Herbst (1892), it has been recognized that lithium has at least two distinct effects on echinoderm eggs. It produces exogastrulation (*i.e.*, separation of endodermal from ectodermal and mesodermal structures with essentially complete differentiation of all three) and inhibition of development, which may produce either inhibitory ectodermal or endodermal modifications. Perhaps a third effect is endodermalization of prospective ectoderm. If we can attribute these separate effects to different physico-chemical causes, I should postulate that the primary exogastrulation is brought about by a physical effect of the lithium ion through some such mechanism as the Teorell diffusion effect acting on oöplasmic segregation, accentuating the separation of ectodermal and endodermal factors responsible for differentiation. The secondary inhibition of development could be assumed to be due to an inhibitory effect of lithium on certain enzyme systems (however, contrast Pease, 1942).

There are a number of unexplained effects of certain other external agents in embryonic development. As a working hypothesis, it might be possible to invoke the Teorell diffusion effect as the causative mechanism and plan experiments to further elucidate these phenomena. For example, the effect of blood externally applied when inducing parthenogenesis in unfertilized frog's eggs by pricking needs further investigation. It is well known (Bataillon, 1912) that the presence of blood materially increases the percentage of haploid embryos reaching an advanced developmental stage. Tyler (1931) has described radially symmetrical parthenogenetic embryos of *Urechis*. He suggests that these received a diffuse activating stimulus, rather than a stimulus from one side (such as that provided in normal fertilization), and leading to formation of a bilaterally symmetrical embryo. Tyler (1941) suggests that parthenogenetic activating agents would best be applied in the form of a gradient. In line with these suggestions, we might postulate that the presence of the blood modifies the diffusion gradients set up through the point of puncture of the pricked frog's eggs, or modulates the stimu-

lus to produce more normal embryos. One possible experimental test of this hypothesis is the study of the action, under similar circumstances, of various large molecules with isoelectric points near those of the several blood components.

Utilization of the Teorell diffusion effect as an explanation of the mechanism of localization of certain substances within the ovum does not necessarily imply that the diffusion agent enters the cell from outside. A diffusion gradient of substances leaving the cell, or diffusing from one region to another within the protoplasm, could produce a similar effect. The original polarization of the ovum, laid down during the early growth stages in the ovary, presumably sets up a polar difference in metabolically significant substances. This is one of the primary tenets of the axial gradient theory of C. M. Child. The products directly or indirectly resulting from these differences in metabolic activity may be free to diffuse from regions of higher to those of lower concentration. In so diffusing, under certain conditions, a Teorell diffusion effect may be established, thus secondarily inducing a movement of other charged particles. Gene products, diffusing from the nucleus or chromosomes during certain periods of cell activity, may similarly bring about movements of other substances.

A SPECIAL CASE: *Styela partita*. While the scheme outlined above (in terms of a polar defect) might account for segregation of materials along the polar axis, it could not, without addition or modification, account for a more complex type of oöplasmic segregation, such as that found in the egg of *Styela* and described by Conklin (1905). Upon fertilization of this ascidian egg, there is a primary segregation of materials resulting from a downflow of the yellow and clear substances from the animal toward the vegetal pole (Conklin, 1905, Figures 1-6). This active migration is completed within ten minutes after the entrance of the spermatozoon. Then the sperm nucleus moves to one side in the lower hemisphere, inaugurating a secondary segregation of materials to form the posterior yellow crescent (Conklin, 1905, Figures 8, 9, 13, 14, 15). Opposite this crescent, at the future anterior region of the egg, the light gray crescent arises. As a result of segregation, the animal hemisphere is occupied by clear protoplasm and the remainder of the vegetal hemisphere by dark gray yolk. In line with the polar defect theory outlined above, it would be tempting to suggest that the sperm entrance point serves as a second point of entrance for a diffusing substance which sets up the secondary bilateral pattern of the embryo, but such an explanation is inadequate. As Conklin (1905) demonstrated, the sperm nucleus does not always take the shortest path to the equator, but appears to move in a certain meridian. This seems to indicate that the path of the spermatozoon is determined by the structure of the cytoplasm. We must, thus, assume a bilateral orientation predelineated in the "framework" of the ground substance. Therefore, Harrison's (1945) diagram of the

pattern of the ovum must be modified to include bilaterality as well as polarity, in order to be applicable to the case of the unfertilized *Styela* egg. The situation obtaining in the unfertilized *Styela* egg may perhaps best be visualized in terms of a bilateral liquid crystal structure extending throughout the cell (FIGURE 14), serving as the "framework" of the

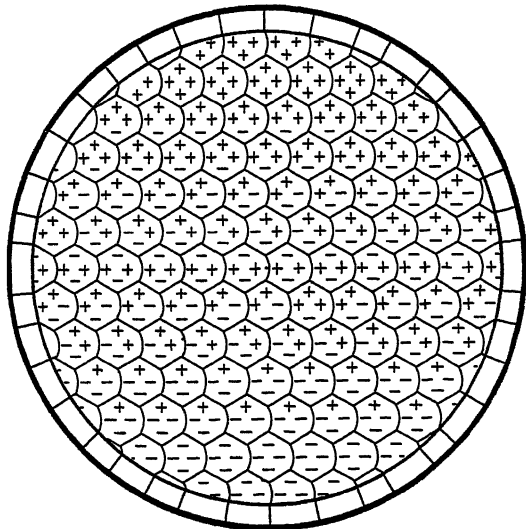


FIGURE 14. Diagram of hypothetical structure of an ovum, modifying HARRISON'S (1945) text figure 12 to include a cortex and a basic pattern of bilaterality in the lattice of the ground-substance. The lattice also provides a structural arrangement for the polarity of direction, while the distribution of the two kinds of particles (designated by + and -) within the interstices of the lattice indicates the polarity of stratification.

protoplasm, and leading to a directed diffusion. If directed diffusion leads to the establishment of a particular pattern of oöplasmic segregation, perhaps we may dispense entirely with the polar defect hypothesis. It is probable that segregation takes place in both the polar and antipolar fragments of cut *Cerebratulus* eggs. The antipolar fragment lacks the polar defect. Possibly, a liquid crystal structure with polar and bilateral orientation provides the requisite conditions.

Undoubtedly, different patterns exist in the unfertilized eggs of different species of animals. In the frog's egg, it is probable that bilaterality is not determined until the time of fertilization. In the eggs of ascidians, it is apparently predetermined in the unfertilized egg. In the eggs of mollusks and annelids showing spiral cleavage, bilaterality may be determined at the time of fertilization (Just, 1912; Morgan and Tyler, 1930), but there is presumably an asymmetry of structure leading to spiral cleavage of the egg or its fragments, which is determined at an early precleavage stage.

The Significance of Oöplasmic Segregation. No more striking example of the significance of oöplasmic segregation can be found than

in the case of differentiation without cleavage in the egg of *Chaetopterus*. After a short exposure to certain solutions (mixtures of $2\frac{1}{2}$ molar potassium chloride and sea-water) both fertilized and unfertilized eggs pass through certain well-defined phases of segregation, as described by Lillie (1902, 1906) and by Brachet (1937). The yolk accumulates as a dense mass in the interior, and other granules or vacuoles assume a polar or peripheral position. FIGURE 15* shows a photomicrograph of one of

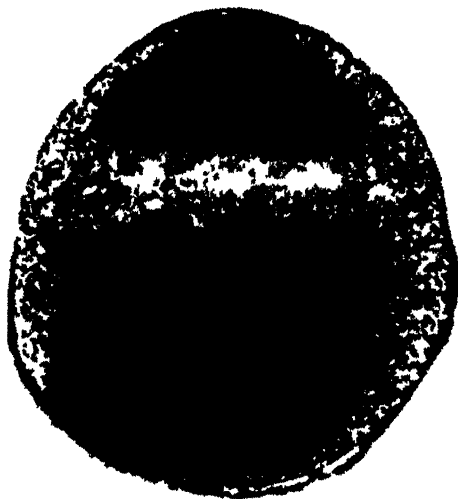


FIGURE 15. Photomicrograph of an unfertilized egg of *Chaetopterus pergamentaceus*, treated with a mixture of 2.5 M potassium chloride (10 parts) and sea-water (90 parts) for 64 minutes. Photographed 3 hours and 45 minutes after treatment. Note marked segregation of cytoplasmic components. A few hours later, cilia were differentiated at the surface. Magnification 660 \times .

these embryos, just before the differentiation of cilia. There is a remarkably clear-cut segregation of parts. Subsequently the peripheral protoplasm becomes ciliated and more vacuolated, so that the embryo resembles, in part at least, a trochophore with inner yolky endoderm, ciliated surface and equatorial band of vacuoles. Lillie's (1902) Figure 8 shows a normal trochophore and his Figures 1-7, etc., show the ciliated structures which develop without cleavage from unfertilized eggs. Embryos differentiating without cleavage do not develop an apical tuft. Lillie correlates this with the fact that the defect in the ectoplasm, where the endoplasm comes to the surface, is obliterated by the artificially induced flowing movements.

In the same cultures,* some eggs are found in which the streaming movements of the interior protoplasm continue after the initial segregation has been accomplished. These amoeboid masses never differentiate

* See footnote on page 670.

the structures so characteristic of the other pseudo-larvae. Therefore, it appears that the proper degree of segregation must be maintained for differentiation to occur.

It is my belief that oöplasmic segregation is of much greater significance in interpreting the development of egg-fragments than has been suspected previously. Studies of the development of egg-fragments, such as the classical experiments of E. B. Wilson (1904) on the egg of *Dentalium*, clearly indicate an association of specific embryonic potency with certain protoplasmic areas. For example, the lower polar area of the egg of *Dentalium* contains the material of the antipolar lobe. This region is definitely related to the formation of apical tuft and post-trochal regions. In *Dentalium*, there is, thus, a very early prelocalization of em-

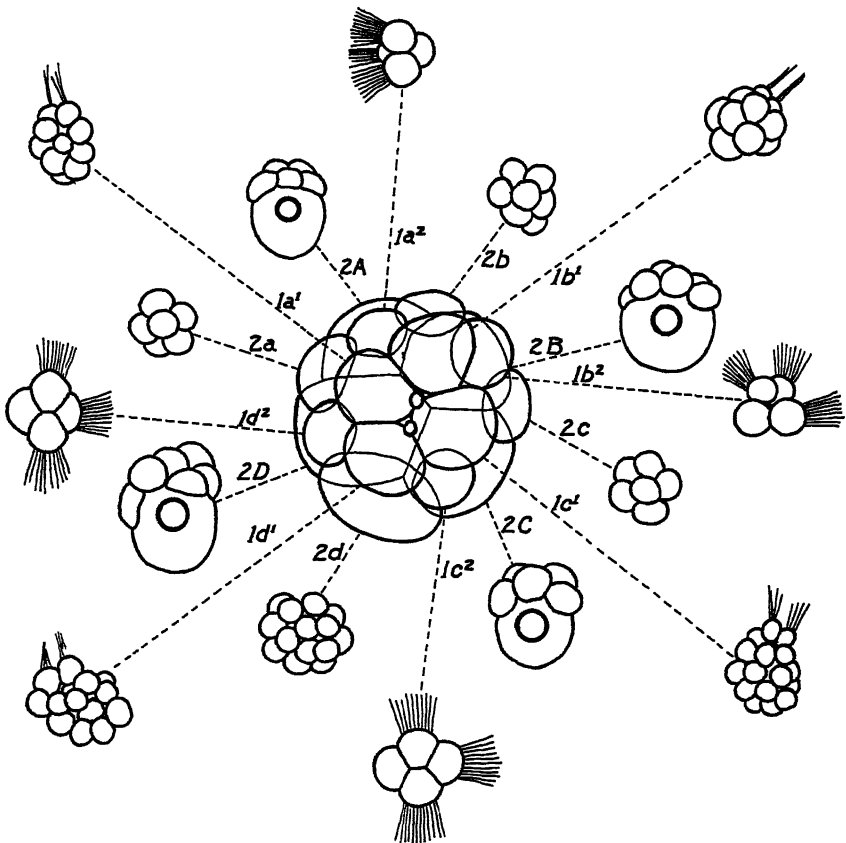


FIGURE 16. Differentiation of the blastomeres of the 16-cell stage of the *Nereis* egg after separation by dissection. (After COSTELLO, 1945b.)

bryonic potency, associated with the very early visible segregation of the lower polar area.

In the egg of *Nereis limbata*, it has been shown (Costello, 1945b) that, from the time of the first cleavage, the isolated blastomeres develop as partial embryos. This is demonstrated very clearly by isolating the blastomeres of the 16-cell stage (FIGURE 16), when only isolated trochoblasts ($1a^2-1d^2$) differentiate prototrochal cilia, only macromeres tend to gastrulate, etc. It is, therefore, of interest to inquire whether this cleavage mosaic is foreshadowed by a prelocalization extending back into the unsegmented egg. Cutting the *Nereis* egg across the equator, shortly be-

TABLE I
DEVELOPMENT OF FRAGMENTS OF FERTILIZED *NEREIS* EGGS
(OBTAINED BY HORIZONTAL SECTION)

	Isolated	Cleared	Ciliated	Number			Anal pigm.
				Gastrulae	Proto. pigm.	Eyespots (1) (2)	
Nucleated (polar)	25	20 (-3 abn.)	16	16	4 (-1?)	1 (+1?)	1
Non-nucleated (antipolar)	25	0	0	0	0	0	0
Whole denuded egg	35	35	35	—	12	6 6	6

fore the first cleavage, produces polar fragments (upper fragments) containing the cleavage spindle, and antipolar fragments lacking spindle

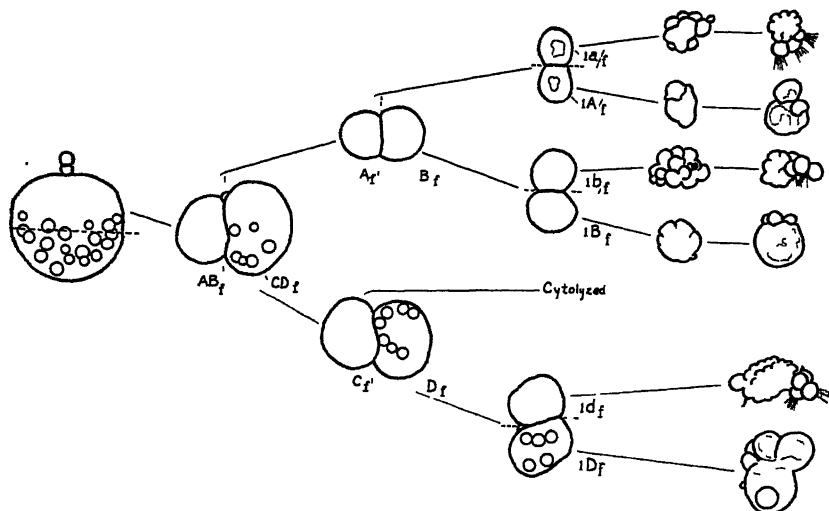


FIGURE 17. *Camera lucida* sketches of progressive blastomere separation of the cleavage products of an egg fragment of *Nereis*. The row of figures at the extreme right indicates the final products of differentiation of the surviving isolates. Compare with progressive blastomere separation of whole egg (Costello, 1945b, Figure 4).

and nuclear materials. Only the polar fragments cleave (TABLE I) and may produce essentially complete embryos (Costello, 1940a). Isolated

blastomeres of these egg-fragments develop in essentially the same manner as isolated blastomeres of whole eggs (FIGURE 17), clearly indicating that the prototrochal material cannot be at the extreme lower pole of the fragment, but occupies a position corresponding to its position in the whole egg (Costello, 1940b). Originally, I suggested three alternative hypotheses (FIGURE 18) to account for this result. First (FIGURE 18, a),

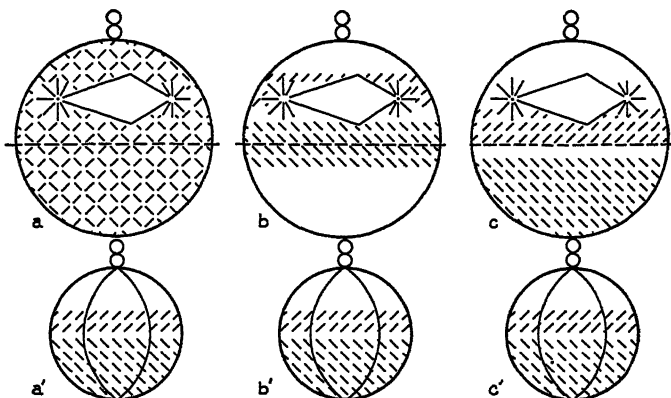


FIGURE 18. Diagrams of three alternative hypotheses concerning distribution of materials in whole eggs and egg-fragments (see text).

it is possible that there is no prelocalization of the materials for prototroch formation (/) and for gastrulation (\) just prior to the first cleavage, when the egg-fragments were obtained. Secondly (FIGURE 18, b), it is possible that these materials are already segregated, but are segregated in the animal hemisphere, the lower half of the egg being essentially unimportant at this time. Thirdly (FIGURE 18, c), it is possible that the segregation of potencies has already occurred, but that the animal fragment is capable of regulating and produces the missing potencies out of other materials than those originally destined for these parts. I am now inclined to view the first hypothesis as the most likely. Since visible oöplasmic segregation in *Nereis* does not begin until after the formation of the polar bodies and is not well-advanced until just before the third cleavage, it is reasonable to suppose that germinal prelocalization is occurring simultaneously with visible segregation of the formed cytoplasmic inclusions.

If a series of different egg species were studied, I believe that it would be possible to establish a correlation between the time of oöplasmic segregation and the degree of embryonic determination. However, we must bear in mind that centrifuging experiments on many forms have clearly indicated that there is no causal relation between visible particles, displaceable with centrifugal force, and morphogenetic values. For example, some of Spek's beautiful figures of *Nereis* eggs stained with neutral red and Nile blue sulfate show a striking "bipolar differentia-

tion." My own studies have shown that, when these stained *Nereis* eggs are subjected to an appropriate centrifugal force (FIGURE 19), all stained

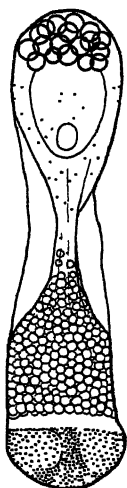


FIGURE 19. *Nereis* egg centrifuged 61 minutes at 66,000 times gravity. Sketched immediately, before change of shape. The strata, in order, proceeding from the centripetal pole, are: oil layer; hyaline zone surrounding upper portion of germinal vesicle; indistinct layer of fine granules, which stain with neutral red; broad stratum of yolk spheres; a second, narrow hyaline zone; centrifugal zone of heavy granules, including vortex of granules which stain deeply with neutral red.

materials are sedimented into definite strata. Such eggs may nevertheless develop normally. The "acid" and "alkaline" regions are not regions of acid or alkaline ground substance (hyaline protoplasm) but are regions in which the contained granules or vacuoles stain differentially. So far as we can ascertain by observation of stained and unstained eggs, there are no visible differences in the *hyaline protoplasm* of the different regions.

The conclusion that the visible cytoplasmic components of invertebrate eggs have no morphogenetic value has recently been questioned by Raven (1938) on the basis of experiments on the eggs of *Nereis* and *Chaetopterus*. Raven and Bretschneider (1942), using low centrifugal forces on the eggs of *Limnaea*, have objected to the conclusions of Conklin (1910) and Clement (1938). However, their objections appear to me to be without adequate foundation, since they ignore the fact that Clement (1938) obtained normal development of *hyaline fragments* of centrifuged *Physa* eggs. Harvey (1946) recently obtained plutei from the clear quarter of the *Arbacia* egg.

The apparent paradox between the results on the development of egg-fragments and of centrifuged eggs admits of easy solution. We need only assume that the mechanism of normal segregation segregates both *visible* formed inclusions and *invisible* morphogenetic substances. The

invisible morphogenetic substances, such as hormones, enzymes, or the like, become associated with the hyaline protoplasmic base in the interstices between the granules or vacuoles. Subsequent centrifuging might easily displace the large visible particles without displacing the invisible substances which are associated with the protein framework of the cell. This conclusion is basically the same as that of Conklin (1931) resulting from centrifugation of the *Styela* egg. It is not the visible granules that have morphogenetic value, but the special, localized hyaline protoplasm with which these granules are normally associated.

The Teorell diffusion effect theoretically provides us with a mechanism which might bring about both a visible stratification of the suspended oöplasmic substances and a parallel but invisible segregation of the "formative stuffs." The primary requirement is that both types of materials be charged.

It has been suggested by Weiss (1939) that diffusible substances (such as inductors or hormones) can act only to bring out differences already existing in an embryo. He uses as an analogy the photographic developer which does not create the picture, but merely converts a latent image into a visible one. Weiss states (pp. 441-442), "It is inconceivable that a chemical agent diffusing indiscriminately through a body whose parts are all alike should ever be able to produce local differences. . . . the problem of organization can expect no elucidation from the study of the 'dark-room' phase of the process." I should like to point out here that a diffusible substance which possesses a difference of mobility of its dissociable parts could act, *via* the Teorell effect, to bring about a *segregation* of other charged particles and thereby actually create a pattern where none previously existed.* Two diffusion gradients of different types, originating from points 90 degrees apart, might give a more complex pattern. An interaction between the components of this pattern, once a certain threshold of concentration has been reached, might produce still more complex patterns.

Another example of a pattern which can be produced by diffusion of a substance into a gel lacking a preformed structural basis for the pattern is the well-known Liesegang phenomenon of colloid chemistry. When two solutions, capable of forming a relatively insoluble precipitate, are allowed to interact inside a gel, the precipitate, under certain conditions, is deposited in layers which are repeated in regular fashion. These are the Liesegang rings, or Liesegang stratifications. The explanation of this phenomenon, which involves diffusion in relation to the degree of local supersaturation, can be found in any good textbook of colloid chemistry.

* Obviously, diffusion from one side, or directed diffusion, is not *indiscriminate* diffusion. But one could visualize a pattern of segregation arising if an egg rests with one surface against the substratum, inhibiting diffusion from this side, and permitting free diffusion into the free surface from the external medium (*vide* work on the *Fucus* egg, summarized by Whitaker, 1940). The diffusion of the amphibian organizer from the chorda-mesoderm into the overlying ectoderm is no more indiscriminate. Organizer action, which appears to depend upon the competence of the reacting tissue, is probably a typical example of "developer action" similar to that described by Weiss for hormones.

In view of the lack of data on the physico-chemical conditions within the developing ovum, the diffusion effect theory of oöplasmic segregation is, at present, only a working hypothesis. I have suggested this hypothesis, not in the belief that it explains all the facts, but rather in the hope that a more adequate explanation of oöplasmic segregation may be evolved. It is hoped, furthermore, that this presentation may rekindle interest in the classical materials of experimental embryology, the eggs of the marine invertebrates.

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Discussion of the Paper

DR. ROBERT CHAMBERS (*New York University, New York, N. Y.*):

I was interested in Dr. Costello's introducing a diffusion gradient model as a hypothesis to explain orientation in an egg. I only wish he could have taken time to present more, and in greater detail, from the great wealth of his own experimental observations.

Concerning the existence of a polar differentiation which persists from the ovarian egg, I wish to mention the egg of the *Cerebratulus*. The excentrically placed germinal vesicle maintains the same relative position as that of the nucleus of the cells of the germinal epithelium, while the micropyle, which is an interruption of the vitelline membrane, as it expands on contact with sea-water, lies at the opposite pole where the epithelial cell had separated from its substrate. Hence, under normal conditions and in the absence of external disturbing factors, the polar bodies arise almost exactly opposite the spot where the sperm is most likely to enter. Insemination can occur anywhere over the surface of the egg, but the growing aster, once the sperm has entered, brings the sperm-head into the position where nature had originally intended it to be. Can it be that the gelating monaster, with the streaming entailed in its growth, has something to do with a reorientation of previously dislocated basic patterns? We know that the development of totipotent egg-fragments is also preceded by the formation of a monaster. May not this phenomenon produce the required polar orientation in each fragment?

DR. D. P. COSTELLO:

Theoretically, it seems possible, as Dr. Chambers suggests, that the streaming movements associated with the growth of the sperm aster may orient substances within the egg, to give a polarity of stratification, though not a polarity of direction. The latter is probably laid down in the egg during its earliest oögonial history. However, if we postulate this activity as a characteristic of sperm asters, we encounter the difficulty of explaining orientations which bear no constant relation to the position of the sperm path, as has been pointed out for the case of the secondary bilateral segregation in the egg of *Styela*. Rashevsky has suggested the possibility that the centrioles of asters are diffusion centers, bringing about an orientation of chromosomes in the mitotic figure, but there is no direct evidence for this. He has not discussed centrioles in relation to oöplasmic segregation.

DR. A. M. SHANES (*New York University, College of Dentistry, New York, N. Y.**):

Dr. Costello has suggested that Teorell's theoretical approach to the "diffusion effect," devised to account for ionic gradients, is applicable to the distribution and movement of particles in the fertilized egg. It may be desirable to call attention to a limitation of the theory as developed by Teorell and its possible significance.

Teorell assumes that diffusion potentials set up by the continuous diffusion of ionized substances are responsible, in the steady state, for the differential distribution of ions on either side of any boundary which serves to support such diffusion gradients. Unfortunately, under conditions of rather high electrolyte concentration such as exist within most if not all cells, such diffusion potentials would be insignificant. Thus, Dr. Osterhout set up a model in which CO_2 served as a source of diffusing ions across a non-aqueous layer. A differential distribution of ions occurred, but the expected potentials arose as ionic changes proceeded rather than before. The potentials were obviously the result rather than the cause of the ionic transfer, and this even though hydrogen ions (the most mobile and therefore the best from the standpoint of large diffusion potentials) were involved and their gradient very high.

Dr. Osterhout's results are easily interpreted from the standpoint of the Donnan equilibrium, for bicarbonate ions were retained within the "cell" while hydrogen ions exchanged with other "extracellular" small cations. Thus bicarbonate constituted the indiffusible ions required for a Donnan equilibrium; the ionic gradients and associated potentials would thus develop as actually observed.

It is difficult to see, in view of such results, how a diffusion effect could cause the movement of microscopically visible particles, particularly with the speed and over distances as great as those which have been

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described for some eggs. Even if molecules as small as proteins were concerned, the porosity of the protoplasm necessary for their migration would eliminate the possibility of ion retention such as seems to be required for the diffusion effect.

THE ORGANIZATION OF THE AMPHIBIAN EGG DURING FERTILIZATION AND CLEAVAGE

By G. FANKHAUSER
Princeton University, Princeton, N. J.

Introduction

IN contrast to the beautifully transparent or semi-transparent eggs of invertebrates and ascidians reviewed by Costello, where processes of oöplasmic segregation may be followed under the microscope, the eggs of amphibians are perfectly opaque. Following fertilization, one can detect sufficient changes at the egg surface to be sure that more important rearrangements of materials must be taking place inside. However, when the egg is fixed and sectioned, one merely sees a heavy suspension of yolk platelets, with a small amount of cytoplasm scattered among these presumably inert materials. There is little to attract our immediate attention and to arouse our curiosity.

It is no wonder that the study of the organization of the amphibian egg in fertilization and cleavage was long neglected. Well in the foreground during the early days of *Entwicklungsmechanik*, in the hands of Born, Schultze, Roux, and Spemann, it was pushed into the background by the sensational success of the transplantation method that may be applied to the egg in slightly more advanced stages. During the past few years, there has been a revival of interest in the initial stages of development. Obviously, to trace the origin of the already complex organization at the blastula and gastrula stages is as important a task today as it ever was (*cf.* Harrison, 1945).

The unsatisfactory state of our knowledge of the egg in its earliest stages is vividly portrayed by the fact that the revival of interest at once led to an animated controversy between different workers. Dalcq and Pasteels assume a relatively simple organization of the ovum at fertilization which becomes gradually more complex during cleavage. Lehmann, on the other hand, is convinced that the condition of the unsegmented egg is very similar to that of the beginning gastrula, with little change during segmentation. Although the two viewpoints appear to be fundamentally different, they are likely to converge as more facts become known.

To make matters worse, the professional language of the embryologist is full of ambiguous and, perhaps, outmoded terms that are bound to foster misunderstandings. Several eminent investigators have warned us against the dangers inherent in the use of such expressions as "determination," "segregation," "organ-forming substances," "organizers," etc.

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The list of terms "the use of which is not recommended," to borrow Needham's phrase (1942), is growing all the time and may soon include most of those that have become dear to us through long association and, therefore, seem to be full of meaning, although it now appears that this meaning may not be exactly the same for all workers in the field. It seems doubtful that the solution to these problems of vocabulary will be found in the introduction of a whole series of new terms. While some, if generally accepted, may help to clarify, others are bound to add to the confusion. As far as the organization of the ovum is concerned, the primary need is for more and better established facts of sufficient convincing power to bring about more general agreement among different workers on the factual level.

Under these circumstances, it may be most profitable to review the overall picture of the organization of the amphibian ovum as it stands today, to point out the few accomplishments and the great gaps in our knowledge that still exist. Such a review should comprise (1) the observations on the visible organization of the egg, including both nuclear and cytoplasmic phenomena; (2) the experimental tests of the invisible organization of the cytoplasm; and (3) the results of experimental analysis of the nucleus in so far as they concern our problem.

Visible Organization of the Amphibian Egg

Nuclear Phenomena. At the very beginning of its career, the egg of almost all salamanders passes through a crisis which might well prove to be fatal if it were not for the existence of a special compensating mechanism. In frogs and toads, fertilization is normally monospermic. If, under laboratory conditions, two or more spermatozoa are allowed to enter the egg, the supernumerary sperm nuclei divide independently, at the same time as the diploid fusion nucleus. Cleavage is abnormal, leading to the formation of haploid, diploid, and mixed cells, and development comes to an end in embryonic or early larval stages (Brachet, 1910, 1912; Herlant, 1911).

In the majority of urodeles, on the other hand, fertilization is normally polyspermic. Up to ten or more spermatozoa may enter the egg, depending on the species. During the first three hours, the internal developments are similar to those in polyspermic frog's eggs and seem to predict certain disaster. However, at the critical time, as the principal sperm nucleus unites with the egg nucleus, the accessory sperm nuclei begin to show signs of degeneration. They may go on to prophase and even release the chromosomes, but the sperm asters do not divide, and the remnants of the accessory nuclei are soon pushed out of the way by the large asters of the diploid mitotic system (FIGURE 1; Fankhauser 1932a; Fankhauser and Moore, 1941a). The nature of the inhibiting factor is not known. It seems to spread from the vicinity of the dominant nuclear system. In any case, there must be a change in the cytoplasm

DIAGRAMS OF NORMAL FERTILIZATION IN TRITON

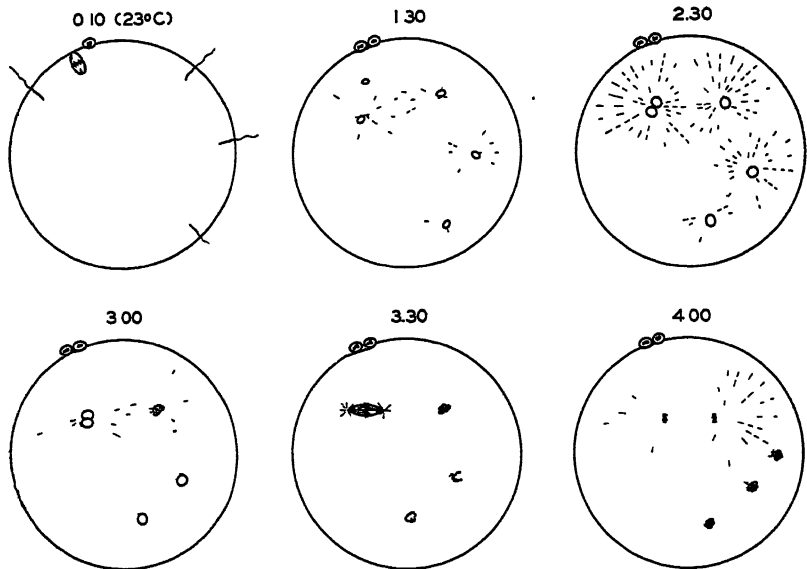


FIGURE 1. Diagrams of polyspermy in the European Newt, *Triton*. The egg is represented in side view, animal pole up; the nuclei, maturation spindle, and cleavage spindle at metaphase are greatly enlarged relative to the size of the whole egg. The asters are not enlarged.

10 minutes after insemination (at 33°C.): metaphase of second maturation division, penetration of four spermatozoa.

1 hour 34 minutes: second polar body given off, small egg nucleus moves toward nearest sperm nucleus which will become the principal sperm nucleus. All accessory sperm nuclei develop normally. Differences in the size of the sperm asters are related to the amount of active cytoplasm present in the animal and vegetal hemispheres.

3 hours 20 minutes: egg nucleus and principal sperm nucleus in contact. Maximum development of sperm asters. Accessory sperm nuclei still normal.

3 hours: fusion of egg and principal sperm nucleus; fading of sperm asters. Two small asters appear in center of principal sperm aster. Accessory sperm asters remain undivided. Accessory sperm nucleus nearest fusion nucleus shows signs of degeneration.

3 hours 34 minutes: metaphase of first cleavage mitosis. All accessory sperm nuclei degenerating. Note eccentric position of cleavage spindle.

4 hours: early telophase of first cleavage mitosis. Note growth of asters at poles of first cleavage spindle which tends to center the mitotic figure and to push the remnants of accessory sperm nuclei out of the animal hemisphere.

surrounding the accessory sperm complexes which blocks their division.

We easily forget that both nucleus and cytoplasm are also involved in the fulfillment of another prerequisite of normal development, *viz.*, the establishment of a mitotic apparatus that will assure normal cleavage of the egg. Under normal conditions, the cycles of the nuclear and cytoplasmic components of the mitotic system are perfectly coordinated. However, if we prevent the union of the egg nucleus and the principal sperm nucleus, by dividing the fertilized egg into two parts, or by eliminating one of the nuclei, the chromosomal and centrosomal cycles are frequently thrown out of gear. In fragments of salamander eggs, and in whole eggs following the removal of the egg chromosomes, the isolated

sperm nuclei show a whole series of abnormal mitotic phenomena: absence or inactivity of the division center leading to a monaster; delayed division of the center; precocious division producing multipolar figures; and, finally, the cytoplasmic component may go through the paces all alone and still be able to induce division of the cell body (FIGURE 2; Fankhauser, 1934a; Fankhauser and Moore, 1941b).

BEHAVIOR OF SPERM NUCLEUS IN ANDROMEROGONY IN *TRITON*

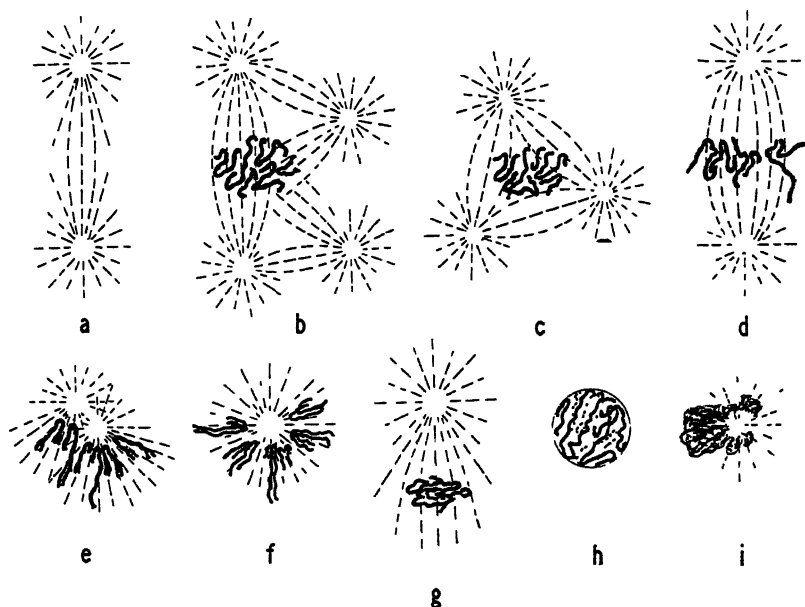


FIGURE 2. Diagrams of various types of mitosis found in androgenetic egg fragments of *Triton*. In the absence of the egg nucleus, accessory sperm nuclei may divide. Frequently, the chromosomal and centrosomal cycles are out of gear. Several types of mitoses may be found in a single egg fragment.

a, division of aster in complete absence of chromosomes.

b and c, precocious division of sperm aster producing tetra- and tripolar figures.

d, normal mitosis.

e, delayed division of aster, at a time when the chromosomes had oriented their attachment points toward the single aster. Whole (split) metaphase chromosomes are distributed irregularly between the two poles.

f, typical monocentric mitosis (monaster); aster remains undivided, chromosomes divide normally.

g, abortive monaster (chromosomes fail to orient their centromeres toward the single pole and do not divide).

h, sperm nucleus in prophase, no aster (later stages of "anastral" mitosis of sperm nuclei have not been found).

i, degeneration of sperm nucleus without attempt at division.

The egg nucleus, when isolated in a fragment of the fertilized egg, is in a more difficult position since it is not normally associated with an active division center. A centrosome may arise, but it usually fails to divide. If no centrosome appears, the egg nucleus may remain in late prophase and grow to extraordinary size, or it may divide repeatedly by an anastral mitosis that looks as if it had been borrowed from a plant

cell (FIGURE 3). It is important to note that this anastral form of mitosis also may induce cleavage (Fankhauser, 1937). Cell division itself, the

BEHAVIOR OF EGG NUCLEUS IN GYNOGAMETOGENY IN *TRITON*

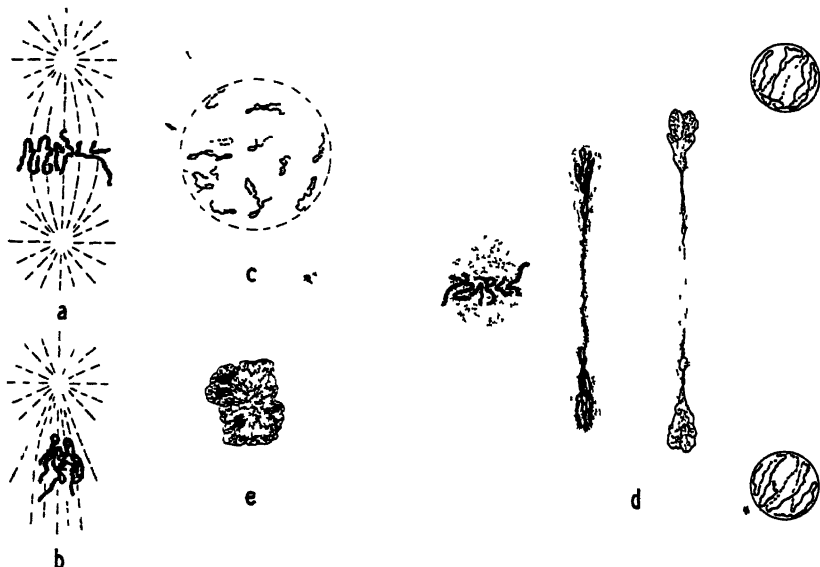


FIGURE 3. Diagrams of types of mitosis in gynogenetic egg fragments of *Triton* (fragments of fertilized eggs containing the egg nucleus alone).

- a, normal mitosis of egg nucleus (very rare).
- b, abortive monaster.
- c, "permanent" prophase with continued swelling of nucleus (twelve chromosomes, the haploid number in *Triton*, may be counted).
- d, four stages in anastral mitosis: *metaphase*: with short "spindle" formed by "half-spindle components" derived from nuclear sap; *anaphase*: stretching of spindle material, incomplete separation of chromosomes; *telophase*: disappearance of spindle material (note similarity to amitosis); *prophase* of following mitotic cycle.
- e, degeneration of egg nucleus without attempt at division.

most basic process of development, is an example of a phenomenon that was known, in the days of classical developmental mechanics, under the perhaps inadequate but illustrative name, "multiple assurance."

In this connection, another observation should be mentioned which clearly shows how particular conditions in the ooplasm may control the behavior of the nucleus. Following A. Brachet's (1922) discovery of the peculiar reactions of the spermatozoa in immature sea urchin eggs, Bataillon (1929, 1934) described similar phenomena in eggs of *Hyla* and *Triton*. In immature eggs which contain the first maturation spindle, the spermatozoa do not form vesicular nuclei associated with large asters. Precociously, small chromosomes are released which are located on a single aster or in a small truncated spindle simulating the maturation spindle. The mitotic figures remain blocked in this condition until the egg dies. It is interesting that the same reaction may be obtained in

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mature eggs if these are first made "immature" again by a two-hour exposure to carbon dioxide.

Cytoplasmic Phenomena. The primary polarity of the egg is clearly expressed in the ovarian eggs of most amphibians in the distribution of superficial pigment and of the yolk platelets. From what we know of the origin of the animal-vegetal axis in eggs of invertebrates, it seems likely that it is present in the very young oocyte but initiated, or at least re-oriented, by some factor in the ovarian environment. It should be emphasized, however, that the existence of such an external factor has never been demonstrated satisfactorily, contrary to the statements made in some textbooks.

Dorso-ventral polarity, or bilateral symmetry, becomes visible in many amphibian eggs after fertilization, when the grey crescent, which marks the position of the future dorsal lip, appears on one side, at or below the equator. Ancel and Vintemberger (1933) have shown that, in *Rana fusca*, the formation of the crescent is connected with extensive shifts of materials at the egg surface.

In some species, even unfertilized eggs may show bilateral symmetry very clearly. According to Pasteels (1937), certain egg batches of *Rana esculenta* show an oblique pigment boundary, higher on the future dorsal side. The same phenomenon had been described earlier in the axolotl, by Banki (1927, 1929). During the formation of the grey crescent, the whole egg seems to rotate, raising the dorsal side still further. Ancel and Vintemberger (1933) claim that this "rotation" is a purely cortical phenomenon and does not involve the whole mass of the egg. Banki (1929) also applied vital stain marks to the axolotl egg immediately after fertilization. Those on the ventral side remained localized, those on the dorsal side spread in the direction of the median plane during the formation of the grey crescent.

If we are, thus, certain that bilateral symmetry may be present in the egg before fertilization, we also know that the median plane may still be shifted after insemination. In some species, like *Rana fusca*, the position of the point of entrance of the sperm may have an important orienting influence, so that the grey crescent in most cases forms on the opposite side of the egg, as shown most recently by Ancel and Vintemberger (1938d). In other anuran species, there is no constant relation between the point of sperm entry and the median plane (*Rana esculenta*, *Discoglossus*: Pasteels, 1937, 1938). In urodeles, polyspermy would prevent a simple relationship. Even in selected monospermic eggs, the planes of fertilization and of bilateral symmetry do not coincide (*Cryptobranchus*, Smith, 1922; axolotl, Vogt, 1926; Banki, 1927).

The final position of the median plane may also be changed by an artificially imposed rotation of the egg. Ancel and Vintemberger (1938a) placed unfertilized eggs of *Rana fusca* on a slide in an oblique position, with the vegetal pole raised 135° . After fertilization and expulsion of

the perivitelline fluid, the eggs could respond to gravity and rotate until they reached the normal equilibrium position. When the grey crescent appeared, it was almost always located on the "descending side" of the egg, *i.e.*, on that side over which the vegetal hemisphere descended during equilibration of the egg. The same result was obtained when the rotation was imposed as late as 60 minutes after fertilization. Beyond this time, it was without effect (Ancel and Vintemberger, 1938b, c). Further experiments showed that the effect of the first rotation may be reversed by a second rotation in the opposite direction, and that the orienting influence of the spermatozoon may be canceled by subsequent rotation (1938e). The observations were extended to *Rana esculenta* and *Triton alpestris* (1938f) and, for *Rana esculenta*, confirmed by Pasteels (1938). There is no doubt that, during the first hour following fertilization, an artificially imposed rotation of the egg can reorient the plane of symmetry. Later on, as the grey crescent begins to form, this treatment is no longer effective. It seems as if an easily displaced substance had now become fixed in a certain area.

Microscopical study of sections through eggs before or after fertilization has long been neglected. Banki (1929) cut axolotl eggs in half along the median plane and saw a broad cortical zone in the animal hemisphere, surrounding a brownish central mass, while the vegetal hemisphere was largely occupied by white, heavy yolk. During formation of the grey crescent, the cortical zone spread out considerably on the dorsal side and formed a thin layer.

More recently, Lehmann (1941, 1945) cut formalin-fixed eggs of the axolotl in two and described a ring of pigmented, sub-cortical or "marginal" plasm in the animal hemisphere which he identifies with the marginal zone of the gastrula, *i.e.*, with the presumptive notochord-mesoderm area. This conclusion is based on the observation that the ring of marginal plasm may be slightly wider on one side. The investigation seems to be of a preliminary nature and should be extended to eggs of various species of amphibians before it can support far-reaching theoretical conclusions.

Undoubtedly, it would be much easier to understand the organization of the egg at fertilization if it could be traced to its origins during the growth period in the ovary which, in amphibians, may last for weeks or months. A highly promising beginning has been made with the aid of histochemical tests, particularly in the hands of J. Brachet (1944). The so-called plasmal reaction, first applied to eggs of axolotls by Voss, indicates the distribution of a special type of phosphatide which is frequently associated with ribose-containing nucleoproteins. In the very young oocyte, the reaction is limited to the "yolk nucleus," a concentration of mitochondria, which later breaks up and forms a peripheral ring. It is interesting that the loci of synthesis of both lipids and proteins coincide with the areas containing plasmalogen.

Ribose-nucleic acid is always present in the nucleoli of the germinal

vesicle; in young oocytes, the whole cytoplasm gives an intense reaction; later on, it is largely limited to a perinuclear ring. The germinal vesicle of the full-grown oocyte shows a high concentration of sulfhydryl compounds in the nuclear sap. Following the breakdown of the nuclear membrane, these compounds occupy roughly the same area as the ribose-nucleic acid. These substances spread out from the animal pole, descending, at first, more rapidly on one side, which seems to be the future dorsal side. At the beginning of gastrulation, they are concentrated particularly at the animal pole and in the dorsal lip of the blastopore. It is very tempting to connect the peculiar distribution of ribose-nucleic acid compounds with the origin of bilateral symmetry, although Brachet himself strongly emphasizes the hypothetical character of such a connection.

Experimental Tests of Invisible Organization of Egg Cytoplasm

Maps of Organ-Forming Territories. Although we are well informed of the prospective significance of the various regions of the blastula and gastrula, corresponding maps of the unsegmented egg have not been published so far, perhaps because of technical difficulties involved in applying vital stain marks at sufficiently early stages, before the cortical shifts described by Anel and Vintemberger begin. Such maps would be extremely valuable in the analysis of the rearrangements of materials that take place after fertilization and during early cleavage, as is clearly shown by Banki's observations and by the studies on ingression which are discussed in detail in the paper by Dr. Nicholas.

Effects of Gravity and Centrifuging. The effects of gravity on amphibian eggs have been studied repeatedly since the days of Pflüger, Schultze, and Born, who turned frog's eggs upside down and forced them to develop in this inverted position, either by compressing them between two slides, or by preventing the swelling of the jelly and the formation of the normal perivitelline space. The resulting embryos were abnormal, largely because of disturbances of gastrulation which often began at two different points. The observations have always been rather difficult to interpret because we do not know in sufficient detail how the various substances within the egg are redistributed under the action of gravity. Pasteels (1941), who recently inverted eggs at the time of fertilization, also seems to have limited his observations largely to living eggs. On that basis, he constructed optical sections which indicate that the variable results may be explained by varying degrees of descent of the heavy yolk. In most cases, two blastopores are formed; no normal embryos appear, probably because gravity alone is unable, in the time available, to assure a complete reversal of the original orientation.

However, if inverted eggs are centrifuged at 460 gravities for from one to five minutes, a complete reversal of the original animal-vegetal (= antero-posterior) axis may take place. Gastrulation is unitary, and

about one-half of the resulting embryos are perfectly normal, although their cephalic end corresponds to the original vegetal pole of the egg. The complete reversal of the primary polarity of the egg by mild centrifuging makes it appear unlikely that polarity is determined primarily by properties of the egg surface, since the cortex does not seem to be greatly affected by inversion or centrifuging.

Pasteels (1940b) also centrifuged eggs of *Rana fusca* which were free to rotate and to orient themselves with the animal pole pointing centripetally. Following centrifugation of two to three minutes, at 460 gravities, gastrulation was often normal. In spite of this fact, the axial organs were sometimes completely absent in all or part of the embryo. In some cases, the tail still contained an axial mass of recognizable somites while the trunk was completely unorganized beyond the formation of a mantle of mesoderm. Other embryos were completely anaxial although they differentiated numerous blood cells.

If eggs are centrifuged after formation of the grey crescent, fewer embryos survive, but the resulting tadpoles may be completely normal. The formation of the axial organs, or organogenesis in general, is no longer inhibited. From these observations Pasteels concludes that, at the time of fertilization, the egg contains a substance of relatively low specific gravity which is a precursor of the active principle of the organization center, called organisin. Perhaps this precursor is identical with the ribose-nucleic acid compounds which Brachet found in higher concentration on the dorsal side of the egg. Centrifuged eggs actually show these compounds concentrated at the animal pole. Once the grey crescent has been formed, the precursor becomes fixed in a more cortical area and cannot be displaced by centrifuging.

Pasteels (1940a), repeating still another old experiment, rotated eggs, following the formation of the grey crescent, through 135° and forced them to develop in this position by compression between two slides. The rearrangement of the materials is more uniform than after complete inversion, since the heavy yolk tends to sink down along one side of the egg. However, in its new position, it will have various spatial relations to the old grey crescent area. While the blastopore always appears at the boundary of the heavy yolk mass, its position is also influenced by the original location of the grey crescent. Very often, the point at which invagination begins seems to represent a compromise between these two forces.

These experiments form the basis of a theory of development (Dalcq and Pasteels, 1937, 1938; Dalcq, 1938) which recognizes two important features of the egg at fertilization: an animal-vegetative vitelline gradient, involving the whole mass of the egg, and a dorsal field limited to the cortex. As cleavage proceeds, an interaction between these two components takes place which is visualized as a simple chemical reaction producing different results at different points, because of the varying concentration of the vitelline substance "V" and the cortical sub-

stance "C." The products of this reaction would then create a field of "morphogenetic potentials" which, in turn, would determine the fate of various regions of the embryo.

This theory, which in its details is somewhat more complex than the brief résumé indicates, has the great merit of focusing our attention on the yolk material which is either not as inert as was formerly believed, or always associated with some other, active material. Also, it seems highly probable that interactions between the vitelline gradient and a more superficial dorsal "field" play an important role in the earliest stages of development. The theory, which undoubtedly will be subjected to more experimental tests, has been criticized by Rotmann (1943) and Lehmann (1945). The latter questions the existence of a true yolk gradient in the amphibian egg and claims that distinct masses of heavier and lighter yolk are present. In his opinion, the dorsal field is not cortical, but sub-cortical or "marginal." It is very likely that continued investigations will bring about a *rapprochement* of these conflicting views.

Isolation of Parts of Egg. The invisible organization of the cytoplasm of the egg before and during cleavage may also be studied by isolating parts of the egg, to test their developmental potencies. The interpretation of the results obtained by such methods is somewhat simpler since it may be formulated in terms of the organizer concept.

Spemann (1902) showed that eggs of *Triton* in early gastrula stages may be constricted within the jelly, by means of a loop of fine hair. When the loop divides the blastopore symmetrically, *i.e.*, when the plane of constriction coincides with the median plane of the embryo, complete twins are formed. When the gastrula is constricted in the frontal plane, so that the dorsal and ventral halves are isolated, the dorsal embryo alone develops normally; the ventral one may gastrulate but fails to form any axial organs (somites, notochord, and neural tube).

Similar results are obtained by dividing the blastula or earlier cleavage stages, or by isolating the two blastomeres at the two-cell stage (Spemann, 1901; Spemann and Falkenberg, 1919; Ruud and Spemann, 1922; Ruud, 1925). It is, thus, necessary to conclude that the dorsal and ventral sides of the egg are already different from one another at this early stage. Spemann was inclined to believe that the future center of organization becomes localized on the dorsal side of the egg before the first cleavage, perhaps at the same time as the grey crescent forms in eggs of other species of amphibians, *i.e.*, about one to two hours after fertilization.

However, if eggs of *Triton palmatus* are constricted as early as twenty minutes after fertilization, we still obtain the same results: about one-fifth of the fragments containing the diploid cleavage nucleus give rise to typical ventral embryos (Fankhauser, 1930b), a similar proportion as that produced in experiments on the two-cell stage. The ventral embryos may survive for several days and form mesenchyme, blood cells

and pigment cells, but no trace of notochord, myotomes, or neural tissue ever appears (FIGURE 4). This demonstrates that an important difference

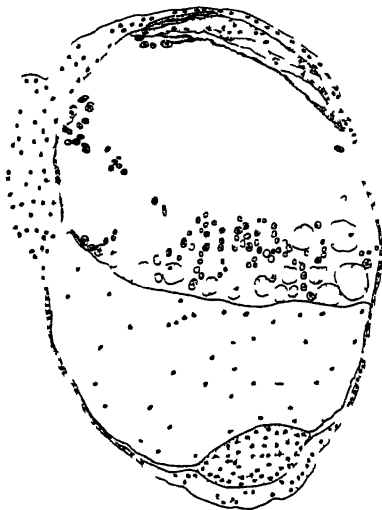


FIGURE 4 Section, along animal-vegetal axis, through typical ventral embryo, developed from a diploid egg fragment of *Triton palmatus* 6 days old (control embryo shows well differentiated neural tube gill buds and indication of forelimb buds. The egg was constricted twenty minutes after insemination. Note presence of mesenchyme near animal pole, blood island (a typically "ventral" differentiation near vegetal pole, irregular thickening of ectoderm, complete absence of axial organs).

in developmental potencies exists between the dorsal and ventral sides of the egg within a few minutes from fertilization. The formation of the grey crescent seems to be a secondary phenomenon which makes this difference clearly visible in the eggs of some species. It is possible that the basic dorso-ventral differentiation is present before fertilization. However, attempts to obtain egg fragments by constriction of unfertilized eggs in the posterior part of the oviducts have not been successful so far.

There is no direct evidence that the dorsal area of the unsegmented egg already has all the properties of the center of organization of the blastula or gastrula, *e.g.*, that, on transplantation, it could at once induce formation of a secondary embryonic axis. Inductions have been obtained by bringing unsegmented eggs or $\frac{1}{4}$ blastomeres in contact with ectoderm of young gastrulae for 30 to 35 hours (Mayer, 1939). However, during this interval, the eggs developed at least to the blastula stage so that it is not possible to ascertain the exact time at which inductive powers arise. As far as the analysis of the cytoplasmic organization is concerned, the fact that the dorsal half of the egg has the capacity to form an organization center while the ventral half does not, is in itself important enough.

Experiments on the two-cell stage have the advantage that both cells

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develop with identical, diploid nuclei and may serve as mutual controls. If the two halves of the unsegmented egg are separated completely, both

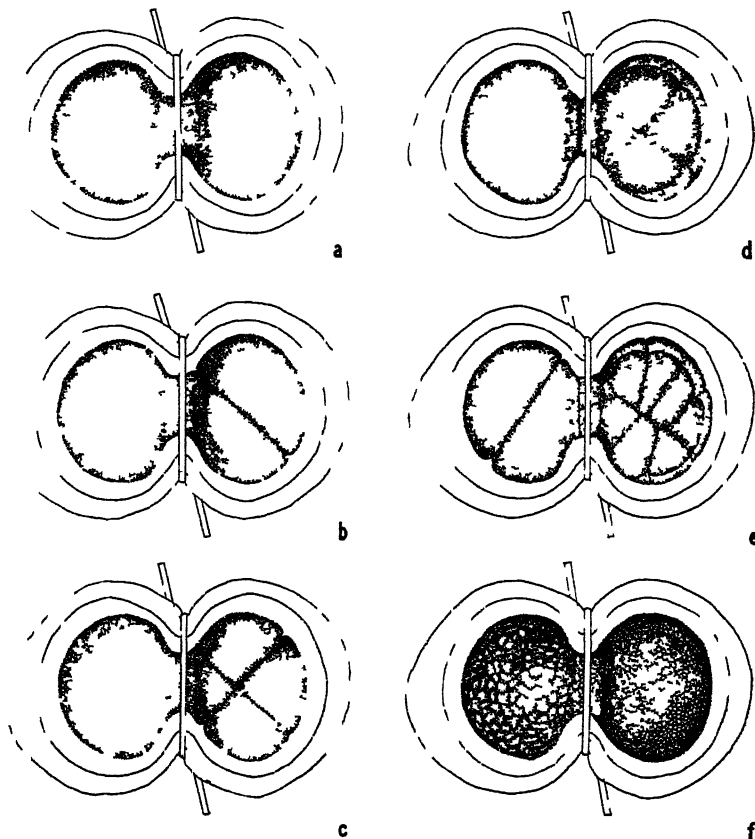


FIGURE 5 Drawings of cleavage of a partially constricted egg of *Triturus viridescens*, illustrating "delayed nucleation" of left-hand part. Magnification about 14 times

a, immediately after constriction (about 20 minutes after insemination). Small black spot in center of light area, to right of hairloop, indicates position of second maturation spindle. Larger dark spot above this area marks point of penetration of a spermatozoon

b, first cleavage of right half containing egg and sperm nuclei

c, second cleavage, one of the four cells connects with bridge, allowing its nucleus to enter

d, third cleavage, the nucleus in the bridge has divided and caused formation of a cleavage furrow to the left of center of bridge. One of the daughter nuclei (16, one of the eight nuclei of the eight-cell stage) has entered the left half

e, fourth cleavage, first division of left half

f, blastula stage. delay in beginning of cleavage of left half is clearly indicated by larger size of the cells

may develop because of the presence of supernumerary sperm nuclei. In rare cases, following constriction in the median plane, one may obtain twin larvae, one diploid, the other haploid. However, for our purposes, it would be advantageous to supply both halves with identical, diploid nuclei. Spemann (1914, 1928) showed that this may be done by

partial constriction of the egg which leads to "delayed nucleation" of the originally non nucleated half. The development of supernumerary

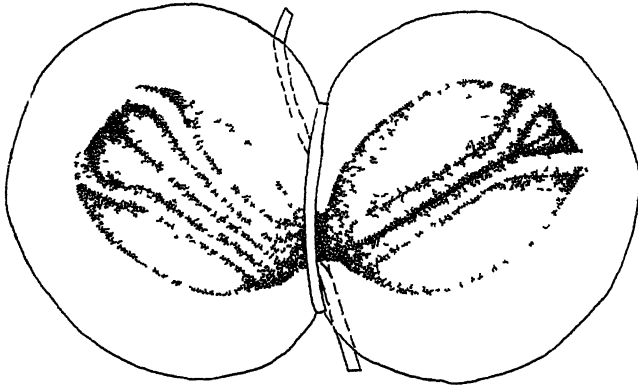


FIGURE 6 Neurula stage of egg that was partially constricted in future median plane, shortly after fertilization. Normal twin embryos. Difference in time of beginning of cleavage of the two halves is still clearly shown. Magnification about 25 times.

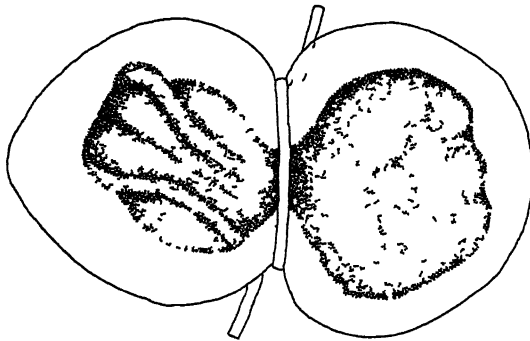


FIGURE 7 Neurula stage of another egg that was constricted in future frontal plane. Dorsal half develops normally, ventral half unable to form axial organs (right).

sperm nuclei is usually inhibited in both halves of a partially constricted egg, as long as the connecting bridge remains wide enough (Fankhauser, 1925, 1930b).

The experiment has been repeated many times with eggs of different species, *e.g.*, *Thiturus viridescens* (FIGURE 5; *cf.* Fankhauser, 1932b). Cleavage begins in the half containing the fusion nucleus. Sooner or later, depending on the diameter of the connecting bridge, a descendant of the original nucleus will enter the bridge and divide there, as may be seen from the formation of a cleavage furrow. One of the daughter nuclei moves into the center of the uncleaved half and initiates a delayed cleavage which proceeds normally. As in experiments on the two-cell stage, one may obtain normal twins (FIGURE 6), or one normal and one ventral

embryo (FIGURE 7). If the connection between the two parts of the egg is maintained during gastrulation, a double-headed monster may result



FIGURE 8 Two-headed larva developed from partially constricted egg, shown in neurula stage in FIGURE 6 39 days old Magnification about 14 times

(FIGURE 8) If the bridge breaks, or is severed artificially by tightening of the hairloop, two normal, though slightly asymmetrical larvae may be produced (FIGURE 9).

Furthermore, following both complete and incomplete constriction of unsegmented eggs, a whole series of intergrades between ventral and normal embryos makes its appearance embryos with weak axial organs, larvae with microcephaly of various degrees (FIGURES 10 and 11), larvae with strong unilateral defects limited to the head or trunk region (Fankhauser, 1930b, 1932b, Streett, 1940). This may be explained by the observation that the plane of constriction may form any angle with the invisible median plane of the egg so that the future center of organization is distributed between the two halves in various proportions. If eggs are constricted very lightly following fertilization, so that they are immobilized within the jelly capsule, the relation between the plane of

constriction and the plane of symmetry may be determined at the time of appearance of the blastopore. Twenty-two eggs of *Triturus virides-*



FIGURE 9 Control larva and twins produced by partial constriction of unsegmented egg in median plane. The connection between the two halves of the egg broke after gastrulation. Note smaller size of eye, balancer, and forelimb bud on right side of left-hand twin, smaller size of left forelimb bud of right-hand twin.

cens tested in this way showed the following positions of the hairloop:

in median plane	3
small angle with median plane	4
oblique	5
small angle with frontal plane	3
in frontal plane	4

On the basis of the results of experiments on the early gastrula stage, where the distribution of the dorsal lip area between the two halves may be observed directly, an egg fragment with a small lateral portion of the organization center would be expected to develop into a micro-axial or microcephalic embryo, while a fragment obtaining approximately one-half of the center would give rise to a "left" or a "right" larva with slight unilateral defects. The similarity of the types of abnormal embryos produced by halves of the unsegmented egg and of the gastrula

raises the question to what extent regional differences in the future center of organization may be present at fertilization.

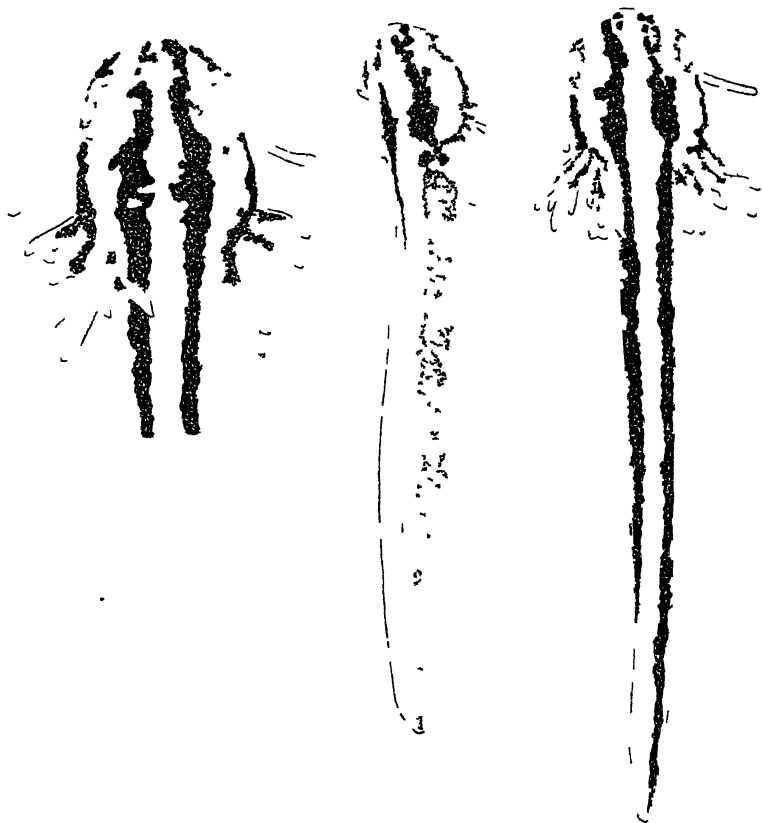


FIGURE 10 Control larva and twins developed from an egg that was constricted in an oblique plane, distributing area of future organization center unequally between the two halves. One twin normal, the other microcephalic and more retarded in development.

One more abnormality must be mentioned. Following median or nearly median constriction of the egg, the "left" twin, characterized by various degrees of underdevelopment of its right side, always has the heart and viscera in their normal position. Among the "right" twins, about 50 per cent show complete *situs inversus*. Reversal of asymmetry occurs whether the division of the egg takes place in cleavage or blastula stages (Spemann and Falkenberg, 1919), or shortly after fertilization (Fankhauser, 1930b). This indicates strongly that the typical asymmetry of the vertebrate body may be traced back to some slight difference in the invisible organization of the right and left halves of the egg at fertilization.

In the early days of experimental embryology, it was customary to

make a sharp distinction between the eggs of Anurans and those of Urodeles. The former were supposed to show more "mosaic" organiza-

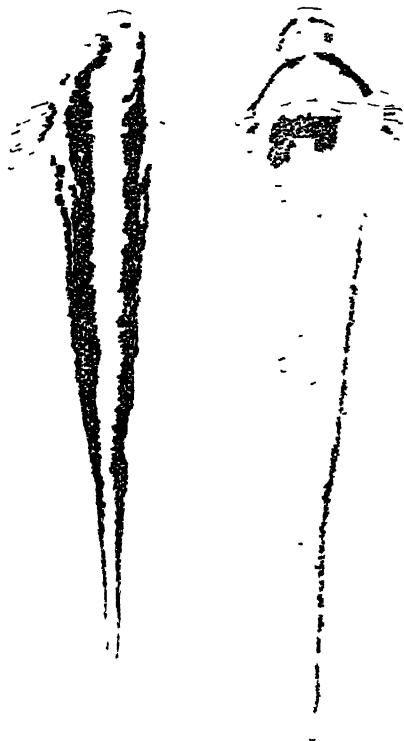


FIGURE 11. Dorsal and ventral views of a single microcephalic larva developed from an egg fragment. The twin embryo was lost. Single balancer in ventral midline, between eyes.

tion, the latter to be examples of the "regulative" type. Although there undoubtedly exist differences in the degree of self-differentiation and of regulation between eggs of even closely related species of amphibians, there is no such clear-cut division between those of the two orders. Once the technical difficulties were overcome, Schmidt (1933), working in Spemann's laboratory, could show that constriction of eggs of *Bombinator* and of *Rana* produces essentially the same results as that of newts' eggs.

While differences along the dorso-ventral axis are well established, those along the animal-vegetal axis have hardly been investigated. We need more information on the potencies of the isolated micro- and macromeres of the eight-cell stage, and of animal and vegetal fragments of unsegmented eggs and of cleavage stages, in extension of the work of Votquenne (1933), Vintemberger (1934, 1936), Streett (1940), and Stablerford (1939). Such experiments are particularly important since tests of the blastula and gastrula stages have demonstrated a considerable

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capacity for self-differentiation on the part of the presumptive endoderm. Furthermore, there is the possibility of an early segregation of some material essential for the formation of the germ cells which, according to Bounoure (1934), takes place near the vegetal pole of the frog's egg before cleavage begins.

Analysis of the Nucleus

A discussion of the organization of the amphibian egg at the beginning of development would be very incomplete without a consideration of the nucleus and chromosomes. In recent years, geneticists have become increasingly interested in the cytoplasm of the egg, for several reasons. First, it is in the cytoplasm that most of the genes produce their effects. Second, presence of certain essential substances in the cytoplasmic substrate in limited amounts may explain some special phenomena, like competition between genes. Finally, the recent work of Sonneborn (1943) and Spiegelmann (1946) and others calls for the presence, in the cytoplasm, of self-reproducing units of nucleo-protein nature, probably derived from genes, which control the synthesis of proteins and enzymes. It is obvious that the embryologist, in turn, can contribute to the synthesis by paying more attention to the possibilities of experimenting with the chromosomes of the egg. I should like to mention briefly some lines of approach that may be followed:

a. Spemann's experiments on partial constriction demonstrated that a single nucleus of the eight- or sixteen-cell stage, which migrates into the non-nucleated half, is sufficient to initiate normal development of that half. Would a single nucleus of a more advanced stage, perhaps of an embryo, still be able to take the place of the normal fusion nucleus? Rostand (1943), repeating an experiment of Bogucki's, smeared unfertilized eggs of *Rana fusca* with embryo pulp obtained from a gastrula, then punctured them with a needle to induce parthenogenesis. Un-smeared control eggs gave the usual small number of dwarfed, unviable, haploid larvae. Smeared eggs produced similar tadpoles, but also a number of more vigorous larvae of normal size which survived for three to six weeks and were possibly diploid. Rostand suggests that, in these cases, a nucleus from one of the gastrula cells on the surface was dragged into the egg by the stylet and took over the functions of the cleavage nucleus, replacing the egg nucleus. It would be interesting to repeat the experiment on a larger scale and to work out the cytological details.

b. We know from observations on androgenetic salamander eggs and egg fragments that cell division may proceed in the complete absence of chromosomes. A blastula may be formed which includes large areas of non-nucleated cells containing actively dividing asters. Such eggs do not survive gastrulation (Fankhauser, 1934a; Fankhauser and Moore, 1941b). Thus, we know that the presence of chromosomes is necessary for normal development from this stage on. Other observations show that the requirements of the egg are more precise. From the time of gas-

trulation on, it needs at least one complete, balanced set of chromosomes. In the absence of the egg nucleus, sperm nuclei frequently form multipolar mitotic figures which result in abnormal distribution of chromosomes. With such varied, unbalanced sets of chromosomes present in different cells, development again comes to a standstill at the gastrula stage (Fankhauser, 1932b, 1934b; Kaylor, 1941).

On the other hand, complete sets of chromosomes may be subtracted or added with relatively slight effect on early development. Essentially normal development to early larval stages may take place in newts and other salamanders with anywhere from one to five sets of chromosomes, in spite of the fact that nuclear and cell size change in approximate proportion to the chromosome number (review in Fankhauser, 1945). Within a wide range of cell sizes, organogenesis is independent of the size of the individual building units.

c. The role of the chromosomes in development may also be studied by suitable crosses between various species of amphibians, some of which lead to an early arrest of development. As an example, we may mention the results of the genus cross between *Triton palmatus* and *Salamandra maculosa*, studied in Baltzer's laboratory by Schönmann (1938) and Lüthi (1938). Development invariably stops at the gastrula stage, when cells begin to die in various regions until the whole egg breaks up, although some cells still appear normal at this time. Signs of a lethal effect may be detected in the early blastula, when chromosomes frequently lag at anaphase and telophase. Obviously, there is some incompatibility between the *Salamandra* chromosomes and their *palmatus* environment which makes further development impossible. However, if a healthy piece of a hybrid gastrula is transplanted to a normal *Triton* gastrula, it may survive and form a harmonious part of various organs, such as the hindbrain and ear, with all the associated cartilages and muscles. Although other interpretations are possible, it seems likely that the cells of the transplant, although still normal at the time of the transfer to the normal host, were destined to die soon because of their hybrid constitution. Following transplantation, some factors residing in the healthy tissues of the host enabled the cells of the graft to overcome their inherent deficiency. Such stimulating effects of the adjacent normal host tissues have been demonstrated conclusively by Hadorn (1935, 1937) in chimaeras composed of parts of unviable merogonic hybrids and of normal embryos.

d. Time does not permit us to discuss in detail the experiments on hybrid androgenesis in which the maternal chromosomes are eliminated following cross-fertilization between two species. One should mention, at least, Hadorn's (1936) now classical experiment because it has a bearing on our interpretation of the cytoplasmic organization of the egg. In an embryo the cells of which contain cytoplasm of *Triton palmatus* and haploid nuclei of *Triton cristatus*, an incompatibility manifests itself soon after the closure of the neural folds, primarily in the head mes-

oderm. All embryos die at this stage (Baltzer, 1930). If a large piece of presumptive epidermis is taken from a still healthy embryo in the gastrula stage and transplanted to a normal gastrula of a third species, *Triton alpestris*, it will survive for months, until the host completes metamorphosis. At that time, specific characteristics appear in the epidermis. In *Triton cristatus*, the surface is smooth, while in *Triton palmatus* it shows toothlike projections formed by rows of flattened cells. The hybrid skin, with chromosomes of *Triton cristatus* and cytoplasm of *Triton palmatus*, shows the structure of the maternal species. This indicates that this characteristic was determined very early, while the egg was still in the ovary, under the influence of the maternal gene complex. The cytoplasm of the egg at fertilization would then contain some element which is responsible for the development of the characteristic skin pattern several months later. The recent work of Porter (1941) and of Moore (1946) on reciprocal hybridization and hybrid androgenesis between different local forms of *Rana pipiens* shows that other characteristics, appearing earlier in development, may also be influenced by the constitution of the cytoplasm of the egg.

Conclusions

The organization of the amphibian egg at fertilization, as it is known today, includes at least the following features:

(1) There must be a basis for the primary animal-vegetal polarity which may be connected, at least in part, with the distribution of yolk and associated substances rather than the properties of the egg surface alone, since the direction of animal-vegetal polarity may be completely inverted by centrifuging.

(2) There must also be a material basis for the bilateral symmetry of the embryo, i.e., the dorso-ventral polarity, which is certainly present within a few minutes after fertilization, and may exist before. The direction of this axis may be shifted by secondary factors, such as a rotation imposed on the egg before the end of the first hour or, in some species, by the point at which the sperm enters the egg. There are indications that the dorso-ventral organization may be connected with the early localization of sulfhydryl and ribose-nucleic acid compounds derived from the germinal vesicle or from the cytoplasm surrounding it. These compounds, in turn, may be essential for the functioning of the future organization center, and for organogenesis in general.

(3) The typical asymmetry of the vertebrate body, which is expressed later in the position of the heart and viscera, also seems to be foreshadowed in the organization of the ooplasm. The nature of this factor is entirely unknown.

(4) The structure of the egg may be still more complex because of the presence of regional differences in the future organization center or in the presumptive endoderm.

(5) There are many indications of early interactions between cytoplasm and nucleus, for instance, the inhibition of the supernumerary sperin nuclei in the urodele egg, the normal coordination between chromosomal and centrosomal cycles of mitosis, and the peculiar reaction of the spermatozoa in immature eggs. All these adjustments are essential for the realization of normal cleavage.

(6) Slightly later, at the time of gastrulation, interactions between genes and cytoplasm assume importance, as is shown by the results of abnormal distribution of chromosomes through multipolar mitosis and by many hybridization experiments.

(7) Observations on reciprocal hybrids and on hybrid androgenesis show that various characteristics, appearing early or late in development, may be influenced by the constitution of the cytoplasm of the egg. The organization of the egg at fertilization may thus be complicated by the presence of components that are responsible for the appearance of such maternal characteristics.

(8) It is doubtful that we shall reach a real understanding of the already complex organization of the egg at fertilization unless we make a determined effort to trace its origins back into pre-fertilization stages, to the growing oöcyte in the ovary. During the weeks or months which the oöcyte passes in its follicle, the foundations of the future individual are laid down while all visible signs point to intense metabolic activity. Even the chromosomes take time out from their preoccupation with meiosis and undergo the profound and peculiar changes resulting in the still poorly understood "lamprush" appearance, because they also are taking part in the synthesis of the elements of a new individual that will resemble the old. The young germ should be in an ideal condition to respond to some of the old questions of the embryologist. To be sure, it is less accessible to experimental procedures than it will be later on, but it may not prove to be as unapproachable as it is commonly supposed to be.

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Discussion of the Paper

DR. ROBERT CHAMBERS (*New York University, New York, N. Y.*):

I wish to continue the point I raised in the discussion of Dr. Costello's paper in which I suggested that the sperm or monaster in many marine eggs may be concerned with the reorientation of formative stuffs. The monaster in the larger and more highly lecithal amphibian egg does not develop to the extent that it does in the echinoderm egg in which the entire egg substance is involved. Is it possible that the lack of a sufficiently extensive aster permits dislocation in these large eggs by fragmentation, or by abnormal sedimentation, or centrifugation to upset the organization pattern?

DR. L. T. STABLEFORD (*Department of Biology, Lafayette College, Easton, Pennsylvania*):

There is evidence from the cytological observations of Schultze, Hibbard, and others that the polarity of the amphibian egg is determined at an early stage of the oöcyte since vitellogenesis is initiated excentrically in that portion of the oöcyte which later becomes the vegetal hemisphere of the egg. With this in mind, I would suggest that the relation between yolk and cytoplasm in the egg is more than "gravitational," that it is, rather, intimately structural.

This concept of a yolk-cytoplasm complex in the vegetal hemisphere runs contrary to the conclusions commonly drawn from inversion and

centrifugation studies. It is usually assumed that, when the egg is inverted, the yolk spherules change position under the influence of gravity and mechanically force a reorganization of the egg. It should be pointed out, however, that this experiment involves, in addition to gravity, the effect of mechanical pressure since the egg must be compressed between glass plates or in a tube to invert it. Granting that Pflüger showed pressure itself to be without effect on the development of the egg, it seems reasonable to suggest that the combination of pressure and gravity disturbs the structure of the egg so radically that the shifted material is actually a yolk-cytoplasm complex.

Further evidence for a yolk-cytoplasm complex as a structural component of the egg comes from the work of Todd in 1940. He indicated that, when the amphibian egg is centrifuged, there is first a movement of liquid material to the animal pole of the egg and that this material flows between the large yolk spherules; it is apparently only when the centrifugal force is strong that the characteristic packing of yolk at the vegetal pole occurs.

I bring up this idea of a possible yolk-cytoplasm complex mainly to call attention to the internal portions of the egg since interest seems to be centering almost exclusively on the cortex or surface coat at present.

DR. G. FANKHAUSER:

In reply to Dr. Chambers, let me say that observations on eggs of several species of amphibians have shown that the fully developed sperm aster is very large and may extend through the entire animal hemisphere. However, the maximum size is not reached until two and a half hours after fertilization (in eggs of newts). While the growth of the sperm aster thus may be an important factor in the rearrangement of certain egg materials, there is no direct evidence that it is instrumental in the segregation of developmental potencies in different parts of the egg.

The views expressed by Dr. Stableford are in general agreement with those developed by Dalcq and Pasteels, who emphasize the active role of the "yolk" in early development. Future investigations of this problem should include a more exact determination of the roles played by the material of the yolk platelets themselves and by other substances that might be closely associated with these bodies.

SIGNIFICANCE OF THE CELL MEMBRANE IN EMBRYONIC PROCESSES

By JOHANNES HOLTFRETER*

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EVIDENCE of the embryological significance of the cell membrane may be derived from a consideration of this structure in connection with the following phenomena: cellular permeability, amoeboid motility, morphogenetic movements, cytoplasmic division, selective adhesiveness, cell polarity, and cellular differentiation. It is clear that such a wide range of phenomena cannot be discussed exhaustively within the frame of the present review. The aim of this paper is, therefore, to give not more than a tentative synopsis of the embryological functions of the cell membrane and to discuss the physico-chemical and physiological properties of this structure only as they may serve this purpose. This paper will mainly be concerned with observations on amphibian development, although it is realized that some of the phenomena to be touched upon have been more thoroughly investigated in other forms, such as the echinoderms. The discussion will include numerous unpublished observations.

Functions of the Coat in the Amphibian Egg

Physico-Chemical Properties of the Coat. The array of protective envelopes investing a fertilized amphibian egg resembles that described for the sea urchin egg by Chambers (1938, 1940). Apart from the external gelatinous layers and the vitelline membrane, the amphibian egg and its subsequent stages are covered by a film, or coat, which is firmly attached to the underlying cell membrane and which seems to be comparable to the hyaline layer in echinoderm eggs. The integrity of the coat requires the presence of small amounts of calcium in the immersion fluid (Holtfreter, 1943a). Amphibian embryos which are placed in an isotonic solution lacking calcium ions fall apart into single cells, and a mucilaginous substance passes into solution. Similar mucus formation and disaggregation occur when the pH of the balanced salt solution is raised above 9.6 or lowered below 4.2, or when solutions of sodium citrate or oxalate are applied. As in the case of disintegrated echinoderm blastomeres, the amphibian cells reaggregate and survive the treatment if, within a restricted period, calcium is restored to the external medium. A new coating substance may be secreted by the outermost surface of the ectodermal and endodermal cells.

* In dedicating this paper to Professor K. von Frisch, I am expressing my great admiration for his scientific work, and my indebtedness to him for the inspiration and kind assistance I received while I was a member of his former Zoological Institute at Munich.

In sea urchin eggs, the hyaline coat evidently consists of proteins. Excess of calcium in the sea water renders this layer rigid and insoluble, while acidulated sea water (pH 3.5) makes it disappear (Moore, 1928). The hyaline layer is furthermore attacked by trypsin, and it is negatively birefringent in the radial direction, indicating the presence of tangentially oriented protein micells (Runnström, Monné, and Broman, 1943). Based upon the data on the susceptibility of the coat in amphibian embryos to acids, bases, and calcium-free solutions, it may be assumed that here, too, proteins constitute the essential components of this structure. Alcohol and other protein coagulants render the coat brittle.

Dissection experiments on living amphibian embryos show that the coat is apparently not a living and indispensable part of the egg and of the epithelia deriving from the egg surface. While covering and interconnecting the peripheral cells as a syncytial layer, the coat, where it forms intercellular bridges, can be drawn out between glass needles into long contractile threads which may be cut off without affecting in any way the viability of the attached cell. The surface of the coat is normally semi-solid and non-adhesive, capable of forming wrinkles when stretched, and being in the living egg in a state of elastic tension. Because of these properties, the coat represents the essential structure which unites the blastomeres into a closely packed body.

As compared with the proximal, uncoated side of the surface epithelia, the coated cell surface is considerably less permeable to water, electrolytes, and various toxic substances. Whereas whole embryos, or embryonic fragments, which are entirely covered by a layer of coated cells can be cultured for long periods in strongly hypotonic media containing traces of calcium, uncoated fragments cytolize in it within a short time. Uncoated cells also show an enhanced susceptibility to hypertonic solutions and vital dyes. It is this reduced permeability of the surface layer which enables the amphibian egg to develop normally in tap water.

A protective coat does not seem to exist in the ovarian eggs of the frog, since isolated eggs of this stage die rapidly in various solutions which do not impair the viability of mature eggs. On the other hand, overripeness is associated with a softening of the egg surface, which is reflected in its reduced resistance to centrifugation and its reduced capacity of closing wounds. It is not unlikely that the abnormal cleavage and gastrulation patterns observed in fertilized overripe frog's eggs (Witschi, 1930; Briggs, 1941) are predominantly due to a partial disintegration of the egg surface, involving probably both the coat and the underlying plasma membrane.

Susceptibility to Mechanical Agitation. This double layer disintegrates readily when disturbed mechanically. The susceptibility can be demonstrated by placing a number of fertilized frog's eggs, with all their coverings intact, in a bag of cheesecloth and letting a quick succession of

water drops hit the external jelly of the eggs. If the drops fall from a height of not more than about 10 cm., the eggs suddenly swell strongly and disintegrate within an hour. It is surprising that the eggs succumb to the rhythmic percussions while they are still enclosed in their protective envelopes and without being visibly deformed by the impact of the falling drops. The observation recalls the early experiments of Dareste (1891) which have recently been taken up by Landauer and Baumann (1943), and which showed that shaking of unincubated chicken eggs tends to produce drastic developmental abnormalities. Fauré-Fremiet (1932) and Battle (1948) found that the eggs of various fishes become rapidly liquefied or develop abnormally when mechanically agitated. Similar liquefying effects of shaking or other mechanical irritations have been observed in Protozoa, bacteria, erythrocytes and other cells (Koelsch, 1902; Angerer, 1936; Chambers, 1924). Frequently, death of the cells is preceded by the formation of hyaline blisters and by considerable swelling, which suggests that disintegration is initiated by a molecular disarrangement of the surface membrane, producing at first increased permeability, and subsequently breakdown (see p. 731).

Significance of the Coat in the Process of Gastrulation. When the cells of an amphibian gastrula enter into the phase of morphogenetic movements, it is in the first place the investing coat which integrates the amoeboid activity of the individual cells into the coordinated and synchronized movements of entire germ layers (Holtfreter, 1943b, 1944). By virtue of this superficial elastic sheet which cements the peripheral cells together into a continuous layer, gliding movements of the cells in any region of the gastrula are transmitted to adjacent regions. Similar effects of a tangential pull can be observed in a layer of ectoderm which is proceeding to close in on a wound inflicted to this layer. It should be emphasized, however, that the motive forces for the gastrulation movements of spreading and invagination cannot be ascribed to the properties of the coat, but originate within the living cells proper. Embryonic cells which are not held together by a syncytial coat are perfectly capable of spreading over an organic or inorganic substratum, and they will slip, singly or in groups, into the depth of a layer of endoderm with which they have been brought into contact. The coat merely regiments these amoeboid movements so that they become collective events. Actually, the tensile strength and contractility of this structure counteract the movements of invagination. Cells which migrate singly into the interior of a gastrula can do so only by detaching themselves from the coat. Where such a detachment does not occur, the invaginating cells become stretched into cylindrical or even filiform bodies, which, through their combined efforts, draw the coated surface inward in the form of an archenteron or other cavities.

It follows from the above considerations that agents which reduce the tensile strength of the coat, or remove the layer altogether, will have

drastic effects on the morphogenetic movements, although invagination will not necessarily be suppressed. Agents having such a dispersive or liquefying effect on the coat have already been mentioned: they are salt solutions which are hypertonic, or free of calcium, or have a pH above 9.6 or below 4.4. The suppressive effect of lithium on the morphogenetic movements in amphibian embryos does not seem to differ markedly from that of other monovalent cations.

A weakening of the coat results in exogastrulation. The movements most inhibited are the epiboly of the ectoderm and the involution of the endoderm, whereas the mesodermal tissues freely invaginate into the interior of the endoderm (Holtfreter, 1933). Ectoderm, when its coat has been removed or has lost its original non-adhesiveness, may even become embedded within the endoderm. Morgan (1903), by applying hypertonic solutions of lithium chloride, obtained such a partial inversion of the germ layers in frog embryos. In a similar way, by making use of the coat-dissolving action of sodium citrate (hypo- to hypertonic solutions), the writer was able to produce frog gastrulae in which the entire "decoated" ectoderm, together with the mesoderm, sank into the interior, while the endoderm became the external layer of the whole embryo.

Exogastrulation can be brought about either by culturing pre-gastrula stages permanently in solutions having only a softening effect upon the coat, or by subjecting the early gastrula for a brief period to media, such as alkali or sodium citrate, which produce an immediate and total breakdown of the coat, then returning them to physiologically normal conditions. The idea of Jenkinson (1906) that hypertonicity of the external medium is not the sole agent which causes exogastrulation, is strongly supported by these experiments where the agents were applied in hypotonic solutions, and where the treatment lasted not longer than 15 to 20 minutes. This gave the disaggregated cells full opportunity to re-establish the continuity of the embryo and to continue differentiating.

It would be unwise to attempt an explanation of the whole exogastrulation syndrome in terms of a weakening or dispersal of the coating substance. However, it is reasonable to assume that the integrity of the coat is at least one of the most important factors safeguarding the normal display of the movements of gastrulation and neurulation.

Fate of the Coat in Later Stages of Development. In consequence of the infolding of all except the prospective epidermal areas of the egg surface, the coat is partly carried into the interior of the embryo and there transformed into the inner linings of the intestinal epithelium, of the neural tube, and possibly of the kidney tubules. In this new position, the surface layer appears to retain its original non-adhesiveness, thereby preventing a fusion of the tubular walls at places where they touch each other. On the other hand, the somite-notochord material which originally forms part of the egg surface seems to lose the coating substance, since after having invaginated it breaks up into tissues which are entirely

adhesive. Pieces of gastrula ectoderm, when grafted into the mesenchyme of older hosts, may either retain their epithelial continuity forming cysts, where the coated surface is turned inside, or they, too, break up into cell strands which have no apparent proximo-distal polarity, are adhesive, and disperse within the host tissues.

During the course of development, the coated surface of the epidermis undergoes a progressive hardening, a process which can be demonstrated by subjecting tissues of different developmental stages to the tension at the interface between air and aqueous media having different surface tensions. Such experiments show that the resistance of the coated ectoderm to being torn apart by the interfacial forces increases considerably after the end of neurulation, and that already in younger stages the cohesion of the coated surface is markedly stronger than that of the inner, uncoated surface of the epithelium (Holtfreter, 1943b). Ectoderm cells which are isolated singly in physiological salt solution acquire, within a few days, an entirely rigid and non-adhesive surface. At the same time, their amoeboid motility disappears, while their ciliary movements continue. This solidifying effect of the external medium is further exemplified in those ectoderm cells which, in a normal embryo, move from deeper layers into the outer surface epithelium, where they acquire the characteristics of coated cells.

It seems reasonable to suppose that the progressive hardening of the epidermal surface is largely a result of the progressive formation of insoluble calcium proteinate, since the length of time required to disintegrate the surface coat in a given concentration of potassium oxalate, of sodium citrate, or of sodium chloride, increases with the developmental stage of the embryo. Swimming larvae can no longer be disintegrated by these methods, indicating an insoluble and possibly denatured state of the surface proteins.

Observations on the Properties and Functions of the Cell Membrane

The eggs of various animals, for instance those of some Nematodes (Spek, 1918) and of the trout (Yamamoto, 1940), before or after the onset of the cleavage process, exhibit undulating or rotating movements of their surface. In the oocyte stage, some eggs, such as those of echinoderms, may form pseudopodia of various shapes, particularly under the influence of penetrating spermatozoa, or of chemicals with parthenogenetic properties (Seifriz, 1927; Runnström, 1928; Runnström and Monné, 1945; Harvey, 1938). Intact amphibian eggs do not seem to perform surface movements although, under abnormal conditions, an unfertilized frog's egg may form local bulges and papillae which tend to become pinched off by constriction (Holtfreter, 1946a). This inertness of the amphibian egg may be the result of mechanical restraint exerted by the relatively tough coat. On the other hand, the uncoated side of the blastomeres, facing the interior of a morula or blastula, is normally endowed with filiform or knob-shaped pseudopodia (Holtfreter, 1943b). With

progressive differentiation, cell motility increases, as may be seen by the morphogenetic movements and by the subsequent migrations and amoeboid changes of the cells. The following data seem to indicate that it is the external cell membrane rather than intracellular structures which are responsible for these amoeboid movements (Holtfreter, 1946-1947).

Structural Organization of Embryonic Amphibian Cells. The architecture of any cell from early amphibian embryos closely resembles the general organization found in *Amoeba proteus* (Chambers, 1924; Mast, 1926) and in *Pelomyxa* (Wilber, 1946). There are four concentrically arranged layers: an inner core of semiliquid "plasmagel," containing the nucleus, the structural cytoplasm, and all of the granular inclusions. Even in a resting cell, Brownian movement causes damped translocation of the granules. This material is enclosed in a wall of "plasmagel," the viscous consistency of which prevents dislocations of particles embedded in it. The third layer consists of the clear "ectoplasmic fluid" which occasionally contains a few granules derived from the granulated endoplasm. The unrestricted translocations which the granules exhibit when entering this layer indicate clearly its highly fluid consistency. The hyaline fluid is surrounded by a well delineated plasmalemma, or cell membrane, which may fold into wrinkles, suggesting a semi-solid consistency.

The thickness of the hyaline layer varies with the composition of the external medium. Under approximately normal physiological conditions, the fluid accumulates only in regions where the cell surface bulges out into a pseudopodium, and it fades out from view where the cell membrane becomes temporarily apposed to the plasmagel (FIGURE 1). How-

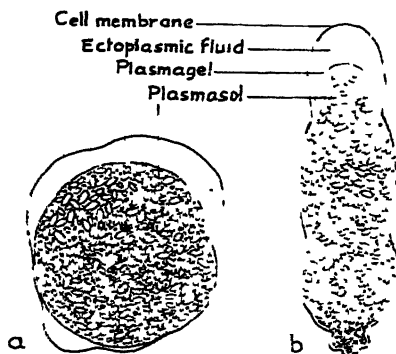


FIGURE 1. Protoplasmic structure of embryonic amphibian cells.

ever, although not visible everywhere, this layer seems to form a continuous shell around the endoplasmic granuloplasm. The continuity can be readily demonstrated when the amount of ectoplasmic fluid is increased following immersion of the cell in isotonic saline solutions lacking calcium, or having a pH between 9 and 10. Solutions more acid, and rich

in calcium, have the opposite effect of reducing the hyaline layer. This agrees with observations on amoebae (Pantin, 1923, 1926) and on amoebocytes of *Limulus* (Loeb, 1928).

The cells constituting the surface of the amphibian gastrula possess a proximo-distal polarity which seems an expression of an inside-outside gradient of the egg. When cells from the periphery of the embryo are isolated mechanically, or by means of coat-dissolving chemicals, the former coated side becomes the posterior cell pole which is characterized by a comparatively stronger contractile power of its surface membrane, by the accumulation of pigment, and by the fact that this side has a reduced tendency to form hyaline protrusions. The uncoated proximal side of the cell becomes the region of pronounced amoeboid activity. It represents, therefore, the advancing anterior pole when the cell is resorting to locomotion. Isolated embryonic cells of any germ layer, but especially those of the neural plate, tend to stretch themselves along their proximo-distal axis into cylindrical bodies having an anterior cap filled with ectoplasmic fluid, while the posterior pole is usually marked as a blackish knob showing surface wrinkles. Following exposure of the cylindrical cell to liquefying agents, such as alkali or sodium citrate, hyaline protrusions also appear along the side walls suggesting that there, too, the cell membrane has remained separated from the plasmagel by a thin layer of fluid (FIGURE 2). With further uptake of water, the ectoplas-

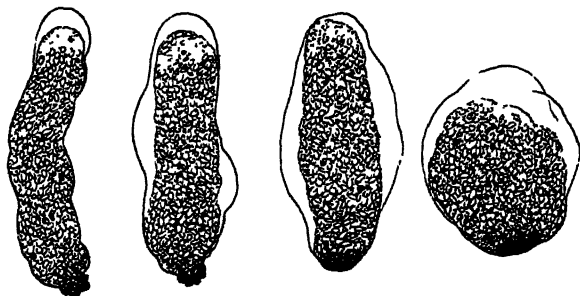


FIGURE 2. Exposure to alkali of a cylindrical cell from the medullary plate causes a lifting-off of the cell membrane from the plasmagel tube and a rounding up of the cell.

mic fluid forms a spacious shell around the entire endoplasm, followed by a rounding-up of the whole cell into a more or less spherical body.

Amoeboid Movements. According to the widely accepted theory of Mast (1926, 1941), form changes and locomotion in *Amoeba* result from contractions of the posterior portion of the plasmagel tube, which push the enclosed plasmasol forward against the plasmagel wall bordering at the hyaline cap of a pseudopod. Subsequently, the frontal gel barrier becomes solated, breaks down, and the endoplasm surges into the fluid of the cap. There, the granuloplasm is presently regelated at its surface, thus forming an anterior extension of the plasmagel tube. The plas-

malemma of the hyaline cap is assumed to be pushed out passively by the hydrostatic pressure of the forward-streaming axial protoplasm. Repetitions of this cycle would result in a continuous transport of the endoplasm from the posterior into the anterior cell portion, i.e., a shifting forward of the whole cell body. Thus, the essential mechanism of locomotion is supposed to consist of localized and reversible sol-gel formations of the granulated endoplasm, with the cell membrane passively yielding to the local variations of the internal turgor.

In elaborating upon this theory, Lewis (1942) assumed that a conversion of the plasmasol into a more viscous state would automatically result in a contraction of this material. Contractions occurred not only in the posterior region of the cell, but Lewis (1933, 1942) observed in lymphocytes and other vertebrate cells that constriction rings passed in regular intervals over the entire cell surface, traveling in an antero-posterior direction. This peristaltic activity was assumed to reside in the plasmagel tube. Lewis concluded that the translocations of the inner protoplasm resulting from these constrictions, together with localized cyclical sol-gel formations, are the essential mechanism of cellular locomotion.

The processes described by Mast and Lewis may occur, likewise, in isolated cells from different stages and germ layers of amphibian embryos. However, the forward-streaming and cyclical sol-gel conversions of the endoplasm observed here, appear to be the consequence rather than the cause of amoeboid movements. Form changes and locomotion of embryonic cells are not necessarily associated with sol-gel transformations of the protoplasm, but seem to be primarily due to alternate expansions and contractions of localized regions of the cell membrane. This conclusion is based upon the following data.

Cellular Motility Associated with Endoplasmic Sol-Gel Formations. Experimental variations of the composition of the culture medium show that both the state of viscosity of the endoplasm and the motility of the cell membrane change with the external conditions, but these two phenomena are not strictly correlated with each other. In a balanced isotonic salt solution ("standard solution") with pH between about 7 and 8.5, isolated cells from the different germ layers of a gastrula or neurula may exhibit the following kinetic phenomena.

Rotating Lophodia in Spherical Cells. At one or several points of the spherical cell the outer membrane is lifted off the plasmagel and forms hyaline bulges which tend to move around the circumference of the cell, but usually avoid the posterior cell pole. After a bulge has attained maximal size, the underlying portion of the plasmagel may liquefy, and the endoplasmic particles surge into the ectoplasmic fluid where they are freely dispersed and thrown about in rapid motion. Subsequently, the liberated particles undergo a closer packing, become almost immobile, and are refurnished with a capsular plasmagel wall lying closely

beneath the cell membrane. Meanwhile, an adjacent region of the cell surface has been growing into a hyaline lobopodium, and the process of solation and subsequent regelation of the inundated portion of the endoplasmic capsule is repeated. Rotating lobopods of a similar kind have been observed in amoebae (Rhumbler, 1898; Jennings, 1904; Pantin, 1923) and in the amoebocytes of invertebrates (Loeb, 1928).

If the embryonic cells are suspended in isotonic saline lacking calcium, or having a pH above 9, they swell considerably through increase of ectoplasmic fluid. A large hyaline cap is formed which rotates rapidly around the surface, while the endoplasmic capsule which is temporarily overflowed by this wave, reacts after a latent period with rapid and merely superficial solations and regelations (FIGURE 3). The belatedness of the endoplasmic eruptions makes it very improbable that this process is the cause of the outbulging and of the rotation of the hyaline lobopod. On the contrary, it seems that the local solations of the plasmagel are of a secondary nature, caused by a liquefying action of the supernatant fluid, the volume of which remains constant throughout the successive cycles of movement. That the cell surface moves independently of the endoplasmic eruptions is borne out by the fact that the larger lobopods consist of several bulges which are separated by notches of contraction, traveling wave-like around the circumference of the cell.

Peristaltic Movements and Locomotion in Cylindrical Cells. The main difference between the pattern of movement in a spherical and a cylindrical cell is that in the latter the hyaline cap and the sol-gel process are confined to the very end of the anterior region. Instead of showing rotating notches of constriction, the elongated cell possesses constricting rings which travel in fairly regular intervals from the anterior to the posterior pole (FIGURE 4). In addition, the elongated cell may perform

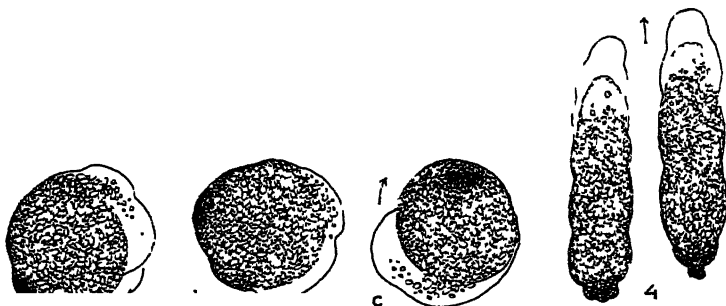


FIGURE 3. Rotating movement and endoplasmic eruptions in a cell isolated from gastrula ectoderm.

FIGURE 4. Peristaltic constrictions and locomotion in a cell from the medullary plate.

bending and twisting motions along its main axis and may undergo longitudinal elongations and contractions of the anterior body portion, while the posterior portion is periodically contracted. This pattern of verniform movements can be exhibited by isolated cells of various germ

layers, although it is most pronounced in the strongly elongated cells from the neural plate.

Since the waves of constriction first appear within the purely hyaline cap, it is clear that they cannot result from a kinetic activity of the plasmagel tube but must be due to an autonomous contractility of the cell membrane. The endoplasmic tube which is, in general, closely associated with the cell membrane seems to be passively indented by the constrictions passing over the cell surface. When a constriction ring approaches the posterior cell pole, the strength of contraction increases, leading to the temporary formation of a small tail knob having a wrinkled surface. The anterior cap never becomes wrinkled but is periodically pushed out in the form of conical bulges. It is these alternating longitudinal elongations and contractions of the anterior region, combined with the periodical shortenings of the tail end which, in the presence of a supporting surface of friction, will produce a shifting forward of the whole cell body. The mechanism of locomotion is thus comparable with that of a creeping earthworm. It is obvious that the peristaltic constrictions, which both in the cell and in the worm occur together with the longitudinal extensions and contractions, cannot be considered as effective movements of locomotion.

It may be mentioned already at this point that the cycle of surface movements may go on indefinitely in the total absence of endoplasmic streamings. Sometimes, however, when a sufficiently large amount of fluid has accumulated in the hyaline cap, the frontal wall of the plasmagel liquefies and the endoplasm flows into the cap where it is presently regulated at its surface. Vital staining of the posterior cell portion shows that, in contrast to the conditions in *Amoeba*, there is no real axial streaming of the plasmasol, but that it is more or less the same circumscribed portion of the anterior endoplasm which is periodically released into the ectoplasmic fluid. This restriction of the gel-sol process to the very tip of the pseudopod is still more conspicuous in the extended processes of more highly differentiated cells.

Cellular Motility in the Absence of a Sol-Gel Cycle. The gelated state of the endoplasm, and hence the frequency of breaks in the plasmagel wall, depend upon the concentration of calcium ions in the culture fluid. Cells which are kept in isotonic solutions of the chlorides of Na, K, or Li, develop a very broad hyaline space, into which the endoplasmic core is almost uninterruptedly releasing its granulo-plasm. Under these conditions, regelation of the liberated endoplasm is considerably delayed. It is completely inhibited if sodium citrate or oxalate is applied, either in addition to the chlorides or in pure solutions. These calcium-antagonizing substances inhibit gelation both in hypertonic and hypotonic concentrations. In the former case (0.5 to 2 per cent solutions) the cells shrink and the hyaline space is reduced to a small blister. The endoplasm underlying the blister lacks the smooth surface of a plasmagel wall, its

peripheral granules being permanently dispersed in the ectoplasmic fluid. Nevertheless, under such conditions, the cells perform amoeboid movements for several hours.

The independence of the surface movements from the state of viscosity of the endoplasm comes out more strikingly when isotonic or slightly hypotonic solutions (0.4 to 0.25 per cent) of citrate or oxalate are applied. The cells swell in inverse proportion to the concentration of the solution, this swelling being confined to the ectoplasmic layer which may eventually separate the endoplasmic capsule completely from the cell membrane. After a rapid succession of local solutions and regelations, the plasmagel wall breaks down all over its surface and the granuloplasm becomes freely dispersed in the surrounding fluid (FIGURE 5). In spite of the total absence of a plasmagel layer, the cell membrane continues performing undulating movements for several hours. This would suggest (1) that membrane motility is less dependent on the presence of calcium in the external medium than is gelation of the endoplasm; and (2) that the surface movements cannot have been caused by contractions, or axial currents, of the endoplasm, but must be ascribed to autonomous changes of the cell membrane.

This total liquefaction and dispersion of the endoplasm is reversible when the cells are returned in time to a balanced salt solution containing calcium ions. Then the granules aggregate into a densely packed body and are surrounded again by a gelled wall which may be clearly distinguished from the fluid hyaloplasm. However, prolonged exposure to citrate or oxalate of hypo- or hypertonic concentrations eventually immobilizes and even dissolves the cell membrane. Disintegration of the membrane begins at one side of the cell and spreads slowly over adjacent regions, while the still intact portion of the membrane fails to contract, indicating that the decomposition of the surface film is preceded by a loss of its original elasticity.

Similar observations have been made on cells which were exposed to alkaline or hypotonic media.

Cell Movements Despite Permanent Quiescence of the Endoplasmic Capsule. The reverse of the conditions in the preceding experiments, namely a permanent gelation of the endoplasmic wall, can be obtained by lowering the pH or increasing the relative calcium content of the culture fluid. Although these treatments simultaneously decrease the amount of ectoplasmic fluid, the amoeboid motility of the hyaline bulges or pseudopods is not suppressed.

Another method of demonstrating the autonomy of the surface movements consists in subjecting the cells to standard solution containing alcohol (10 to 15 per cent). This treatment increases rather than decreases the volume of the hyaline layer, whereas the endoplasm contracts into a quiescent and sharply delineated ball which touches the cell membrane only in the posterior cell region (FIGURE 6). In the com-

pite absence of endoplasmic solations, the cell membrane continues performing the characteristic undulating movements which shift the ectoplasmic fluid over the smooth surface of the endoplasmic capsule. It is mechanically inconceivable that any contractions of the capsule, or endoplasmic currents, might have caused the simultaneous appearance of more than one rotating bulge in the cell surface.

Attention may also be drawn to cells of the type of FIGURES 7-9 which

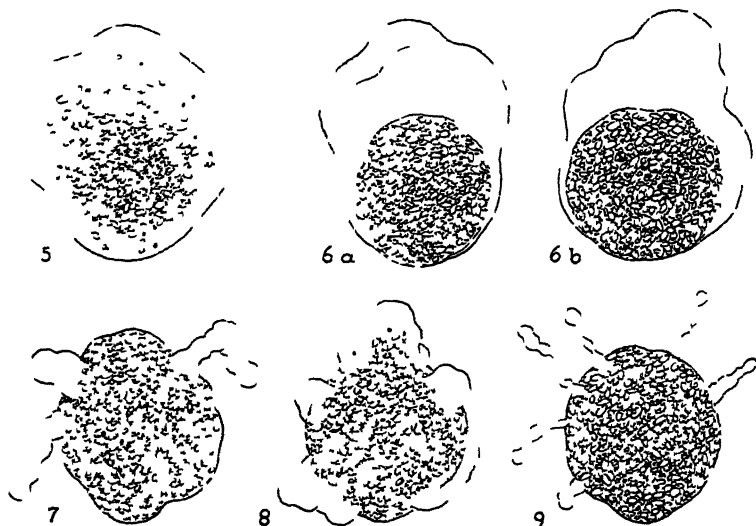


FIGURE 5. Complete solation and dispersal of the endoplasm under the influence of potassium oxalate, while the cell membrane continues to show amoeboid movement.

FIGURE 6. Exposure to 10% alcohol, preventing solation of the plasmagel but not the movement of the cell surface.

FIGURES 7-9. Pseudopod formation and fragmentation induced by brief exposure of the cells to alkali.

show the presence of numerous cylindrical pseudopods, devoid of endoplasm and moving independently of each other. The movements consist in bendings, longitudinal extensions and contractions, and peristaltic constrictions, traveling from the tip to the base of the hyaline pseudopods. These movements cannot be explained by a possible kinetic activity of the encapsuled endoplasm which remains quiescent. Such an outgrowth of the cell surface into numerous blunt pseudopods can be induced by a shock treatment with alkali.

Amoeboid Motility in Cell Fragments Lacking Endoplasmic Structures.

If the conclusion is correct that it is not the reversible sol-gel process of the endoplasm but varying states of contraction of the cell membrane which cause cellular form changes and locomotion, then cell fragments lacking the endoplasm entirely should be motile as well. This is indeed the case.

An effective way of obtaining fragments of the desired composition consists in exposing cells of gastrula ectoderm briefly to alkali, which induces them to spread on the glass surface. After a while, the alkali effect wears off, the flattened cells contract and leave behind on the glass minute portions of their hyaline margin. Since the endoplasm is usually retracted into the bulky cell portion before the peripheral fragments become detached, the latter frequently contain no visible traces of structural cytoplasm. They may survive for many days and exhibit the various kinds of kinetic patterns observed in whole cells, *viz.*, spreading and locomotion of lamellar processes, alternate expansions and contractions of lobose or cylindrical pseudopods, and peristaltic constrictions passing over the surface of tubular fragments (Holtfreter, 1946a). With the aid of a surface of friction, hyaline fragments are capable of creeping forward like a vermiform cell. In cases where a fragment contains a few lipid granules, these move freely within the vesicle, rapidly dislocated by Brownian movement and by the irregular currents resulting from the undulations of the cell membrane. Whenever a portion of endoplasm is present, it forms, within the ectoplasmic fluid, a delineated body having a gelated surface layer and showing but very reduced translocations of the enclosed granules. There occur all intermediary forms between completely hyaline vesicles and others which are almost entirely filled with endoplasmic material. The former are usually much more motile than the latter.

When exposed to increasing concentrations of standard solution, the hyaline vesicles shrink, their motility slows down and eventually stops. This process may be reversed. In still higher concentrations of this solution, the vesicles die and become filled with basophilic granules, showing unrestricted Brownian movement. They can be further dehydrated by various agents such as acids, or concentrated solutions of calcium chloride, basic dyes, or heavy metal salts, all of which transform the vesicle into a thin, granulated film firmly attached to the glass surface (FIGURE 10).

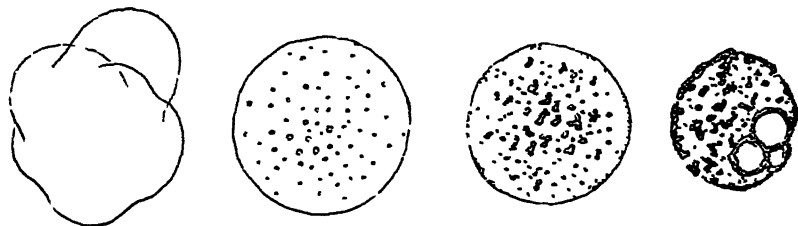


FIGURE 10. Exposure of a hyaline cell fragment to 10^{-1} M CaCl_2 , causing shrinkage, granulation, and collapse of the vesicle in the form of an adhesive basophilic film.

From these observations, it may be concluded that the different modes of amoeboid form changes can be executed by cell portions which lack entirely the inner protoplasmic material capable of a reversible gela-

tion. It appears that this conclusion applies also to *Amoeba*. for Chambers (1924) observed that cut-off pseudopods of *Amoeba* were capable of ingesting food and moving about in the typical amoeboid manner, although they were entirely free from the visible granules of the parent body. Taken together with the evidence of the preceding paragraphs, these data indicate that the endoplasmic gel-sol formation occurring sometimes in whole cells is merely superposed upon the kinetic activity of the cell membrane. The present material furnishes no evidence that the plasmagel capsule has a contractility of its own. Whenever the endoplasmic material changed from a spherical into a cylindrical shape, showing peristalsis, it was tightly enclosed by the cell membrane, suggesting that it was the well-attested contractile power of the latter which had molded the endoplasm. If these two structures were separated by a cushion of ectoplasmic fluid, the outer membrane continued performing amoeboid movements, whereas the surface of the endoplasm remained immobile.

The periodical solutions of the plasmagel apparently result from chemical reactions between the endoplasm and the supernatant ectoplasmic fluid. This process seems to involve the exchange of electrolytes, with calcium ions playing a predominant role in controlling the viscosity of the structural endoplasm. While in some instances liquefaction and dispersion of the granulated endoplasm proceed slowly, comparable to the melting of a submerged sheet of ice containing particles, in other cases the plasmasol may rush rapidly through the opening in the plasmagel wall. This acceleration of flow, in contrast to the liquefaction proper, appears to be caused by the contraction of an external covering. Since forceful endoplasmic eruptions occurred only if the posterior portion of the endoplasm was closely associated with the undulating cell membrane, the contractile force driving the plasmasol toward the hyaline cap is probably furnished by the outer membrane.

Constriction of the Cell Membrane Resulting in Cytoplasmic Division. Isolated embryonic amphibian cells may exhibit three kinds of division phenomena: (a) mitotic nuclear division associated with cytoplasmic fission; (b) division of the nucleus into two or more nuclei without segmentation of the cell body; (c) cellular fragmentation in the absence of nuclear division. The latter phenomenon may occur within a normal embryo, for instance in the flask-shaped blastoporal cells, after they have reached their final destination in the anterior region of the archenteron. The formation of blood platelets has been attributed to the budding-off of cytoplasmic fragments from large mother cells. Lewis (1942) ascribes cytoplasmic division to the same mechanism which produces the constriction waves in migrating cells. The following data support this concept, although they do not agree with the idea of Lewis that it is the plasmagel which performs the constrictions.

In the cylindrical cells mentioned above, it was frequently observed

that a constriction wave became stationary, that the constricting ring cut progressively deeper into the cell body and finally caused a fragmentation of the cell. The products of division were usually very unequal in size and composition. The nucleus remained inert and intact and came to lie in one of the daughter bodies, both of which could survive for several days. That it was again the cell membrane which provided the mechanism of constriction was indicated by observations on the cell fragments mentioned above. Very small hyaline fragments containing neither chromatin nor any other microscopically visible internal structures, would divide spontaneously into two bodies which continued performing amoeboid movements.

Cytoplasmic division in the absence of a nucleus has been observed in the eggs of the axolotl, the second spindle of which had been removed by means of a micro-pipette (Jollos and Peterfi, 1923). Fankhauser (1934) observed in polysperm merogons of *Triton* that cell walls may form around astrospheres lacking chromosomes and that segmentation of the cytoplasm may occur even independently of any nuclear or astral activity. On the other hand, division of the sperm nuclei or of the accessory astrospheres was not necessarily associated with a corresponding cleavage of the cytoplasm. In centrifuged eggs of the sea urchin which had been activated by hypertonic sea water, Harvey (1938) found that cleavage could take place while the egg nucleus was still intact, which suggests that "the cleavage of an egg and the nuclear changes usually accompanying it are quite separate phenomena." Non-nucleate halves of the eggs of sea urchins and of *Chaetopterus*, which had been treated with a parthenogenetic agent, performed amoeboid movements and then fragmented into cells of irregular sizes containing astrospheres but no chromatin (Harvey, 1936, 1938, 1939). In non-nucleate pieces of the starfish egg which had been subjected to parthenogenetic agents, Chambers (1924) observed the budding-off of fragments, some of which possessed no cytasters. There is no observational evidence that the cytoplasmic fissions occurring in bacteria and some algae lacking a distinct nucleus are associated with the presence of cytasters. These observations make it improbable that cytaster formation is a necessary prerequisite for cytoplasmic division.

It would appear that regional differences of composition of the protoplasm, associated with the presence and division of astrospheres, tend to localize and arrest the autonomous constriction movements of the cell surface in an equatorial zone. Here, the constriction would proceed to form a cleavage furrow, perhaps because the underlying cytoplasm is comparatively more liquid and, hence, less resistant than elsewhere, or because some chemical or electric properties of this region induce a locally stronger contraction of the cell membrane. Chalkley (1935) noticed in dividing amoebae that the plasmagel layer in the region of the deepening furrow becomes thinned out, liquefies, and streams away in oppositely directed axial currents. Erlanger (1897), Spek (1918), and

other investigators have recorded the streaming of superficial liquid cytoplasm from the poles toward the plane of cleavage. In centrifuged sea urchin eggs which were subsequently fertilized, the first cleavage furrow sank in more rapidly on the hyaline than on the densely granulated side of the egg (Chambers, 1924).

These observations do not support the view that the viscous plasmagel layer is actively engaged in the cleavage process. According to observations of Motomura (1935) on sea urchin eggs, the external plasma membrane covering the furrow cuts through the viscous cortical layer without dislodging it from the egg surface. Dan, Yamagita, and Sugiyama (1937), from observations on the displacement of kaolin particles attached to the plasma membrane of sea urchin eggs, concluded that the surface area over the spindle poles expands throughout the cleavage process while the prospective furrow region at first shrinks, then increases markedly when it is drawn into the depth of the furrow. Similar conditions apply to the amphibian egg (Schechtman, 1937). However, the opaqueness of the latter egg makes it impossible to watch the behavior of the cytoplasm. That the ingression of the furrow results from the constriction of a densified region of the cell membrane seems to be suggested by the fact that the floor of the furrow is more resistant to chemical and mechanical injuries than is the cell surface over the spindle poles (Just, 1922; Chambers, 1938). This would agree with the conditions in hyaline pseudopodia and cell fragments where it can be noticed that the constricting rings acquire temporarily a higher refractivity than the expanding areas, suggesting alternate states of density of the cell membrane. In dividing fibroblasts, the opposite poles of the cell are known to bulge out into rapidly moving blebs which recall the hyaline blisters formed under the influence of alkali, mechanical irritation, or other agents which weaken and liquefy the cell membrane.

The above considerations make it understandable that external stimuli, causing a local reduction of density and contractility of the cell membrane, will at first increase the permeability of this layer, then induce the formation of pseudopods and hyaline blisters, and finally cause a pinching-off of the out-bulging portions of the cell. This phase may be followed by cytolysis. Most parthenogenetic agents produce cytolysis when applied for longer periods or at higher concentrations. Comparable with the artificially induced formation of hyaline protuberances and their detachment in immature sea urchin eggs (Runnström, 1928), a pinching-off of amoeboid vesicles free of granulated endoplasm may occur in gastrula cells and in early erythrocytes of amphibians which have been briefly exposed to alkali or other agents which at higher concentrations decompose the cell membrane. Mechanical irritation may likewise cause a fragmentation of the embryonic cell. The main difference between the constrictions associated with amoeboid movement and those leading to cell division, or fragmentation, appears to be that the former are reversible and successively involve various regions of the cell surface,

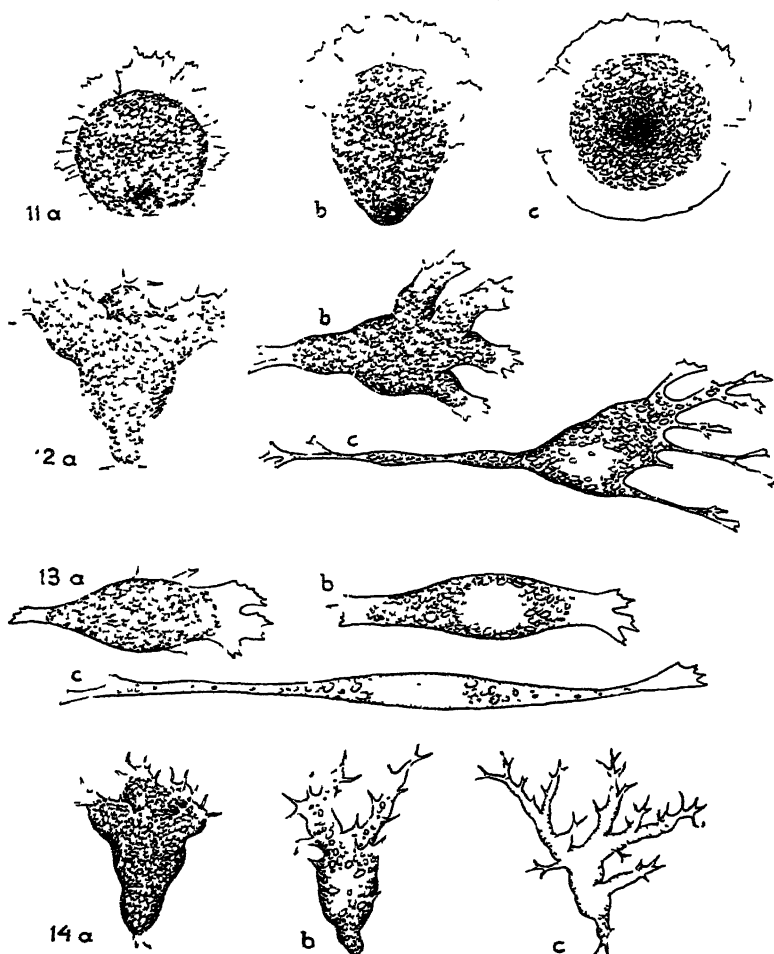
whereas the latter remain localized and cut progressively deeper into the cytoplasm.

Function of the Cell Membrane in the Process of Cellular Differentiation. By way of controlling cellular locomotion and cytoplasmic division, the cell membrane plays an essential role in bringing about the morphogenesis of the different types of cells. A differentiating cell acquires its new shape through the elaboration of new patterns of amoeboid activity, a phenomenon usually accompanied by the development of regional, cell-specific differences of adhesiveness of the surface film. In order to materialize these properties effectively, most cells need an external surface of contact. A few examples may illustrate the situation. The cells to be discussed had been derived from urodele embryos and were cultured singly in physiological salt solution (Holtfreter, 1946b, 1947b, c).

Whereas the different cell types from pre-neurula stages are, after isolation, more or less spherical, showing only slight tendencies of spreading, of becoming cylindrical, or forming slender pseudopodia, their morphogenetic behavior becomes more distinctly cell-specific after they have passed these stages. Isolated prospective epidermis cells from a neurula tend to flatten shield-like against glass or living tissues (FIGURE 11), while the individual cells from the neural plate tend to stretch themselves into long vermiform bodies (FIGURE 4). On the other hand, neuroblasts of the type of Rohon-Beard's cells, as well as the cells from the neural crest, shortly after their isolation from early tail bud stages, adopt a shape somewhat resembling that of an *Actinia* (FIGURES 12a and 14a). In the absence of a supporting substratum, their plump anterior portion may project hyaline tapering pseudopods capable of elongating, contracting, and bending around in curves. The posterior cell portion is conical and can change its shape by way of constrictions and longitudinal stretchings and contractions. These cell-specific differences of kinetic behavior are a reflection of the fact that from now on the various cells are determined cytologically.

Evidently, the different cell shapes arise from the activity of the cell membrane, not the endoplasm. The motile filopods of an early neuroblast contain merely ectoplasmic fluid; the anterior portion of the vermiform neural plate cell may exhibit cylindrical elongations, bendings, and constrictions while not underlain by the endoplasm; the epidermis cell spreads and progresses over the substratum by way of extending a hyaline margin which is distinctly set off from the central body of granulated endoplasm.

With the substitution of lamellar or filiform pseudopods for the earlier rounded lobopods, the amount of ectoplasmic fluid is generally reduced. At the same time, the reversible solation of the plasmagel becomes less frequent and is confined to the increasingly smaller areas which directly border at a hyaline protuberance, while the bulk of the



FIGURES 11-14. Successive stages of differentiation in an isolated epidermis cell FIGURE 11 , in a neuroblast FIGURE 12 , a myoblast FIGURE 13 , and a mesenchyme cell FIGURE 14 .

endoplasm remains unaffected. Local outbreaks of a peripheral portion of the granuloplasm have been observed in the margin of flattened epidermis cells and in the tongue-shaped advancing pseudopods of neuroblasts, myoblasts, and mesenchyme cells. The process is readily recognized because of the sudden acceleration of movement which the granules undergo when pouring into the ectoplasmic fluid. It should be emphasized, however, that, as in the case of lobose or tubular pseudopoda which project freely into the external medium, the flattened pseudopods of attached cells move just as well in the absence as in the presence of gel-sol formations.

The movements of the cell membrane in flattened cells are less co-

ordinated and on a smaller scale than are those in non-attached cells, where they pass in large waves over the whole cell body. Peristaltic constrictions do occur in differentiating neuroblasts, myoblasts, leucocytes, and mesectoderm cells, but they become less conspicuous the more the whole cell flattens against the substratum. Instead, the hyaline margin of an attached cell is engaged in locally independent and irregularly alternating movements of expansion and contraction which produce a constant change in the outlines of the serrated periphery. Furthermore, ring patterns and ruffles may move over the surface of the hyaline margin. Such ruffles are well-known features of the laminar processes in monocytes and macrophages of vertebrates. Fauré-Fremiet (1929, 1930), who observed them in the freely extended membranous pseudopods of choanoleucocytes, stresses the fact that the movements proceed while the centrally located endoplasm remains quiescent. He therefore rejects the idea that the movements of the hyaline processes are caused by variations of the gel-sol ratio of the endoplasm. This conclusion is supported by our observation that the same kind of movements may occur in isolated fragments of the hyaline margin which contain no endoplasm at all. In migrating cells, the endoplasm merely follows the movement of the advancing margin, by filling up successively the hyaline space, thus securing the ground gained by the activity of the ectoplasmic cell portion. If the total sum of periodical extensions outweighs that of contractions, the flattened pseudopod will continue moving forward in one direction.

All cell types mentioned above possess an antero-posterior polarity. The relatively inert posterior pole corresponds to that side which in the blastula stage was facing the outside. In epidermis cells which are merely spreading, the posterior pole takes up an apical position (FIGURE 11c). In migrating cells, the posterior portion is dragged behind and contracts periodically, frequently forming a tail knob, while the advancing anterior region may remain more or less unipolar (myoblasts) or may branch out into several independently moving pseudopods (neuroblasts, mesectoderm cells, leucocytes). If the posterior pole becomes firmly attached to a substratum and the anterior cell portion continues advancing, the whole cell may become stretched into a long ribbon (myoblasts, FIGURE 13c), or only a tail portion is spun out into a fibrous process (neuroblasts, FIGURE 12c). The transformation of the dendritic anterior protuberances of a neuroblast into long fibrous processes is only possible if the bulk of the cell remains sessile and the pseudopods continue advancing by means of their undulating hyaline end-plate. Leucocytes exhibit a pronounced mobility because of lack of adhesiveness of their posterior region and a great agility of their anterior region.

It appears that, with progressive differentiation, the cell membrane becomes more firmly attached to the underlying plasmagel and that this reduces or inhibits its motility. This condition is especially conspicuous in isolated epidermis cells which have been cultured for several days.

Their entire endoplasm forms a gelated layer closely associated with the surface film, which becomes immobile. In fully extended spindle-shaped myoblasts and in dendritic mesenchyme cells, the major part of the cell surface becomes progressively quiescent, motility being confined to the tips of the pseudopods which have remained free of endoplasm.

Congelation of the whole cell occurs in a pronounced form in erythrocytes (Holtfreter, 1947c). Like all other cells of the amphibian embryo, the erythroblasts represent at first globular cells provided with rotating lobopods which may undergo local gel-sol formation (FIGURE 15a). When the hemoglobin becomes visible and the yolk is almost absorbed, the ectoplasmic fluid slowly disappears and the cell membrane settles down over the entire surface of the plasmagel capsule, without, however, being arrested in its kneading movements (FIGURE 15b). Unlike the spreading

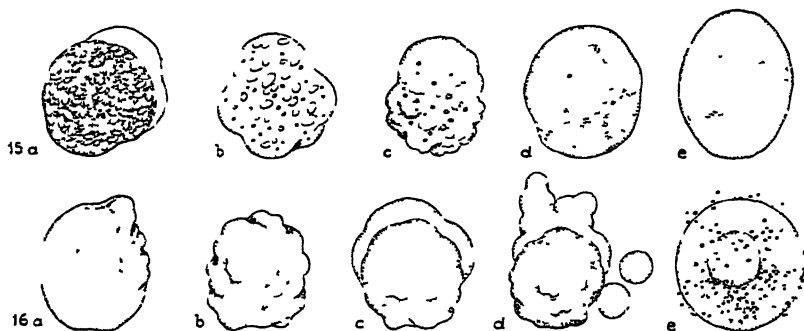


FIGURE 15. Successive stages of differentiation of an erythroblast.

FIGURE 16. Retrogressive form changes of an erythrocyte following exposure to alkali.

movement in epidermis cells, the flattening of the erythroblast into a discoidal body occurs in the absence of any substratum and is executed simultaneously by both the cell membrane and the endoplasm. After the cell has flattened, it remains immobile, and Brownian agitation of the endoplasmic inclusions becomes less discernible. However, the process of immobilization can be reversed, if the early erythrocyte be exposed to liquefying agents, such as salt solutions hypotonic or of high pH (FIGURE 16a-e). Under such conditions, the rim of the discoidal body develops amoeboid protuberances, the cell rounds up and exhibits the kneading movements of its earlier stage, and finally the outer membrane is lifted off from the plasmagel layer and forms once again undulating lobopods filled with liquid cytoplasm now containing hemoglobin. The lobopodia may become pinched off at their base and form mobile fragments (FIGURE 16d). With extended treatment the plasmagel layer becomes liquefied as well, at first locally and reversibly, then all over its surface. This causes the endoplasmic inclusions to be freely dispersed within the now spherically swollen cell. At this stage, the cell membrane becomes immobile and so porous that hemoglobin and even larger particles includ-

ing the nucleus can penetrate it. The final stage of cytolysis is accompanied by the appearance of precipitation granules in the cytoplasm.

A similar sequence of reactions to liquefying solutions can be obtained with other differentiated cells, *e.g.*, leucocytes or mesenchyme cells. The treated cells will return to their earlier mode of motility by replacing the filiform or dendritic processes by large undulating lobopods and they eventually die from being flooded with the immersion fluid.

Thus, cellular differentiation which is associated with partial immobilization and a restriction of amoeboid activity to localized pseudopods, seems to involve a certain amount of dehydration. During the period of consolidation of the final cell shape, the inner cytoplasm may find the proper conditions to develop structures of increasing stability, as, for instance, oriented fibrils. Observational data and concepts as regards the possible genesis of cytoplasmic structures may be found in the publications of Schmidt (1937), Frey-Wyssling (1938), Picken (1940), Schmitt, Hall, and Jakus (1943), and Lawrence *et al.* (1944). If the above considerations be accepted, the appearance of an intracellular cytoskeleton is the consequence rather than the cause of the external form changes in differentiating cells.

Observations on the Composition, Structure, and Physiological Reactions of the Cell Membrane

In order to understand the various functions of the cell membrane, it is necessary to inquire into its chemical composition and physical properties. The pertinent data, although obtained with different methods and on different material, appear to conform sufficiently to be pieced together into a comprehensive, though somewhat sketchy concept. (See the reviews by Harvey and Danielli, 1938; Danielli, 1942; Schmitt, 1941, 1944; Schmitt and Bear, 1939; Monné, 1946.)

Recent investigations afford ample support for the idea, advanced by early workers on cellular permeability, that the cell is bounded by a film containing lipids and probably also proteins. Not only the surface film of the different somatic cells so far studied, but also the outer protoplasmic layer of eggs appear to consist principally of an organized lipoprotein structure.

Runnström and collaborators (1928, 1943, 1945) have shown that, when unfertilized sea urchin eggs are exposed to lytic agents, such as alkali, fat solvents, high temperatures, hypotonic sea water, or merthiolate, their cortical layer bulges out into hyaline blisters and pseudopods which may become detached in the form of "lipoid vesicles." These formations resemble the pseudopods and hyaline fragments in amphibian cells, which arise either spontaneously or under the influence of various cytolytic agents. The cortical layer of the living normal sea urchin egg is positively birefringent in a radial direction, suggesting an ultra-structure comparable to that in myelin formations, where the elon-

gate phosphatide molecules are assumed to form concentrically arranged bimolecular leaflets, the long axes of the molecules paralleling each other and lying perpendicularly to the surface of the body (Runnström, Monné, and Broman, 1943; Monroy, 1946, 1947). Through the action of fat solvents, the birefringence of the cortex may either disappear completely or reverse its sign, becoming a radially negative one. These observations suggest that the cortex contains alternate lamellae of lipid and protein molecules, the latter being extended in a tangential direction and attached laterally to the polar groups of the lipid leaflets (Monné, 1946). It has not been possible to decide whether the entire birefringent cortex, which is quite thick (about 1μ), or only its peripheral portion should be identified with the selectively permeable cell membrane.

Under dark-field illumination, the birefringent cortex of unfertilized sea urchin eggs has a yellow-orange color, changing into white and bluish-grey when this layer is expanding into the pseudopods mentioned above (Runnström, 1928). Monné (1941), who studied the color variations in the protoplasmic constituents of a great variety of cells, concludes that they reflect alterations in the state of hydration of the colloids involved. According to Monroy and Monroy Oddo (1946), the change and final disappearance of color in the cortex of the sea urchin egg is associated with a progressive reduction of the positive birefringence of this layer, indicating a dispersal or dissociation of the constituent lipid molecules, probably because of an increased solvation. It is interesting to note that a disappearance of color and birefringence can be produced not only by fat solvents, hypotonicity and other lytic agents, but also by a slight compression of the egg (Monroy and Monroy Oddo, 1946). Upon return of the egg to normal conditions, the positive birefringence may reappear, although the cortex of compressed eggs fails to recover completely, exhibiting patches of disarrangement of its molecular constituents.

On the basis of these observations, one may assume that the hyaline blisters which can be produced by both chemical and mechanical agents, are the result of a locally increased solvation and porosity of the cell membrane, and the accumulation of water beneath it. In a similar way, the spontaneous local expansions occurring in the cell membrane of amoeboid cells appear to involve a higher state of solvation as compared with that of contracting regions. In a hyaline pseudopod, the out-bulging portions are less refringent than are the constricted ones. Hyman (1917) found that the tip of a pseudopod in *Amoeba* is more susceptible to injurious chemicals than is the rest of the cell surface.

A cortical fine structure comparable to that in the echinoderm egg has been reported to occur in various other cells. Combined chemical, leptoscopic, and polarization-optical studies on the ghosts of hemolyzed erythrocytes suggest that their limiting membrane consists of alternating lamellae of lipids and proteins, their arrangement resembling the

molecular structure of the myelin sheath of nerve fibers (Schmitt, Bear, and Ponder, 1936, 1938; Waugh and Schmitt, 1940). A corresponding lipoprotein structure has been assumed to exist in the cell membrane of living embryonic chicken cells (Hobson, 1941), of differentiated ganglion cells of the frog (Chinn, 1938), and of living erythrocytes and spermatoocytes of invertebrates (Monné, 1941, 1946). According to Fauré-Fremiet (1929), the hyaline pseudopods of choanoleucocytes are birefringent when in a dehydrated and agglutinated condition. Alcohol and ether, in contrast to acetone, destroy this property, indicating that it is due to the presence of phospholipids. I observed birefringence in the surface layer of living amphibian eggs and in the cell membrane of the different isolated cells and hyaline fragments discussed above, but I have not yet succeeded in obtaining more exact data on this phenomenon.

The concept of an organized lipoprotein structure of the cell membrane is supported by a variety of experimental results concerning the permeability, surface tension, solubility, and electric properties of the cell surface (see Harvey and Danielli, 1938). The concept agrees, furthermore, with the findings on the chemical composition of this structure. Monroy and Monroy Oddo (1946), on the basis of quantitative polarization-optical studies, conclude that the birefringent cortex of the echinoderm egg consists predominantly, if not entirely, of phosphatides and cholesterol. In the red blood cell, practically all the lipids are concentrated in the outer membrane, which constitutes the posthemolytic residue (Erickson *et al.*, 1938). According to the analyses of Parpart and Dziemian (1940), this membrane contains lipids and proteins at a ratio of about 1:1.7, the lipids present consisting almost entirely (82 to 98 per cent) of phosphatides, especially cephalin, and of cholesterol.

The presence of phosphatides in the cell membrane of embryonic amphibian cells is suggested by the observation that this structure is readily destroyed by cobra venom. Increasing concentrations of alcohol render the membrane highly permeable and may eventually dissipate it entirely. Although the cell membrane is less susceptible to the absence of calcium ions in the immersion fluid than is the coat of the amphibian egg, its amoeboid activity and stability seem to depend on calcium bound to the lipoprotein components (see p. 711). Monné (1946) draws attention to the fact that the surface of many cells has been found to be rich in calcium and magnesium and that nucleic acids are also present. He suggests that the phosphoric acid groups of the phosphatides and of the nucleic acids are held together by calcium molecules.

Comparison between the Behavior of Lipid Models and the Cell Membrane

At this point, a comparison between the behavior of the living cell membrane and that of artificial films of lipids and lipid mixtures may promote the further analysis of our problem.

Fauré-Fremiet (1925, 1929, 1930) and Runnström (1928) observed certain similarities between the kinetic, chemical, and polarization-optical properties of the cell surface and those of myelin tubes from lecithin or cephalin. Having found that the hyaline pseudopods of amoebocytes are anisotropic and contain large proportions of phosphatides, Fauré-Fremiet (1929, 1930) suggested that their movements could be interpreted as resulting from localized variations in the degree of hydration of surface layers of oriented phosphatide molecules. The extent of hydration and, consequently, the expansions and contractions of the pseudopods were assumed to be controlled by pH and by the kind and proportion of the cations present.

Some gross similarities between the appearances and kinetics of living cells and those of models of lipids and their mixtures with other substances have been noticed by several other workers (Herrera, 1932; Crile, Telkes, and Rowland, 1932; van Herwerden, 1933). It was, however, the aspect of molecular structure and permeability rather than that of motility of the cell surface which led to the more intensive studies on lipid models. Harvey (1912) produced vesicular bodies of lecithin and proteins which reacted to neutral red, saponin, and mechanical pressure not unlike sea urchin eggs. Danielli (1936, 1945) and Harvey and Danielli (1936) studied artificial lipoprotein films from the viewpoint of elasticity, interfacial tension, and adsorption power. In a series of important contributions, Bungenberg de Jong and co-workers (1932, 1935, 1937) investigated the effects of physical agents, of hydration, pH, electrolytes, proteins, and various other substances on the behavior of lipid models. The concepts on coacervation which emerged from these studies represent a very instructive guide for an interpretation of the structural and functional properties of the cell membrane. No less fruitful for the cytologist has become the extensive work on monomolecular layers, which is connected with the names of Adam, Langmuir, Rideal, and Schulman.

The writer has become interested in lipid models because their reactions to various chemical and physical agents seemed to explain some processes connected with Golgi bodies and cellular vacuoles (Holtfreter, 1946c). At the same time, these experiments disclosed phenomena which appear to promote an understanding of the cell membrane. This dual applicability of the models is not surprising since the structure and functions of the cell membrane are supposed to resemble in many ways those of the lipoprotein film surrounding intracellular vacuoles (Searth, 1927, 1940; Bungenberg de Jong, 1932, 1935; Frey-Wyssling, 1938). Some of these observations which have a bearing upon our subject matter will now be discussed. The morphological and kinetic behavior of living cells and hyaline cell fragments was compared with that of myelin bodies from crude lecithin, which were exposed simultaneously to immersion fluids of different compositions. The lecithin used was in an advanced state of rancidity and contained admixtures of cephalin, free

fatty acids, and probably other lipids. This impurity of the "lecithin" preparation was not considered disadvantageous, since it appears that the biological membranous structures likewise represent mixtures of different fatty substances.

When immersed in distilled water or dilute Ringer, the lecithin developed the characteristic myelin tubes and vesicles which, with further uptake of water, grew in length and diameter. The thickness of the tubular walls and the extent of elongation varied with the initial mass entering into the swelling process. The larger bodies usually gave rise to complex systems consisting of vesicles which included other vesicles and tubes of various size. Small grains of lecithin rapidly became spherical and developed a large cavity filled with an optically homogeneous fluid, while the external wall was thinned out into a film having about the diameter of the cell membrane. In their optical appearance (ordinary and polarized light), these small vesicles were practically indistinguishable from living contiguous hyaline cell fragments.

Myelin vesicles, stretched by glass needles into a long tube and released, either contracted or broke down into several portions which rounded up into spherical fragments. When embedded in olive oil, hydrated myelin formations could be drawn out into very thin fibrillar and anastomosing structures resembling the processes of mesenchyme cells. The surface film of a myelin vesicle was capable of sealing up a hole made by a glass needle, and of restoring its continuity when penetrated by vacuoles expelled from the interior.

Reactions of Myelin Bodies to Variations of pH. In analogy with the behavior of hyaline pseudopods, myelin bodies form tubular shapes only within a medium pH range, acidulation causing shrinkage and granulation, alkalization, a vesicular swelling, fragmentation, and dispersal of the bodies. FIGURE 17 shows diagrammatically the relationships between the pH of the immersion fluid and the type of myelin formations which developed at the periphery of a lump of lecithin within the period of an hour. The immersion fluid consisted of distilled water to which HCl or KOH respectively had been added. It will be noticed that the swelling of the periphery increases in direct proportion to the raising of pH. At the lowest levels (pH 2.0–2.2), only a thin surface layer swells into a homogeneous transparent substance. At higher levels, up to about pH 4.0, myelin figures begin to appear, but shrivel up into granules when with further elongation they come into direct contact with the acid medium. Granulation is no longer detectable above pH 4.2. Instead, swelling continues and increases gradually with rising pH. Within the pH range of about 5.0 to 9.0, there are no marked differences in the size and shape of the strongly expanded myelin bodies. At levels above pH 9.4, the tubes become increasingly spherical and may form numerous thin-walled bulges which are pinched off as separate vesicles or may burst and disintegrate (FIGURE 18).

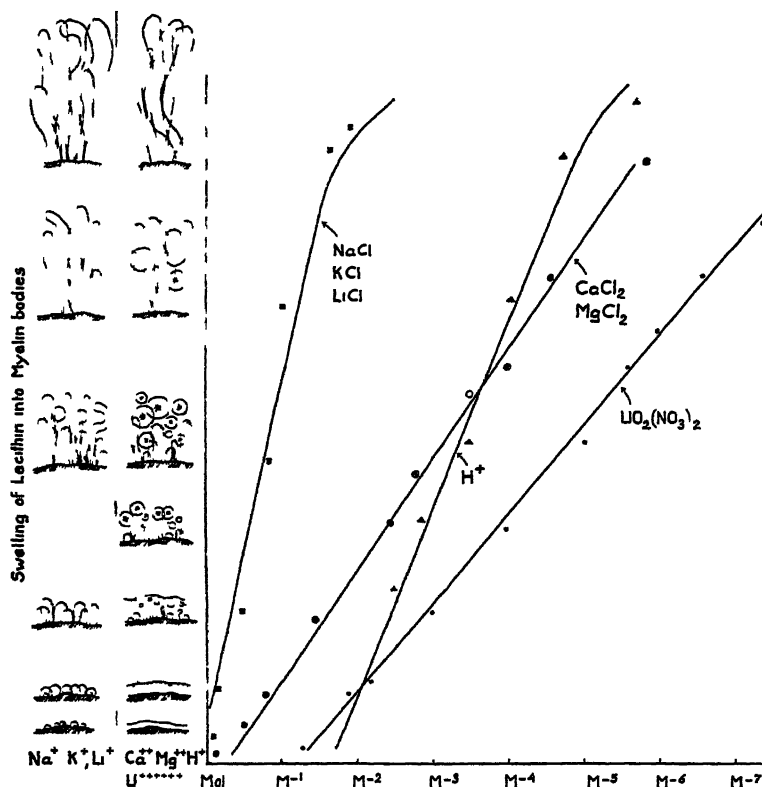


FIGURE 17. Effects of different concentrations of hydrogen ions and various metal ions on the swelling of crude lecithin. The effects have been diagrammatically indicated by the relative thickness of the marginal zone made up of myelin bodies.

When a fully expanded myelin vesicle is transferred from a neutral into an acid medium, it shrinks and the internal fluid gives rise to granules showing Brownian movement. With increasing acidity, the granules become coarser and the vesicle shrivels up and collapses to form a thin, granulated film firmly attached to any contact surface (FIGURE 20a). Adhesiveness of the lecithin vesicles to each other or to glass becomes apparent at about the same pH level (≈ 4.2) when granules are being formed. The extent of flattening of the bodies over a substratum increases with the rate of dehydration.

In an analogous manner, the size, shape and adhesiveness of the hyaline processes in embryonic cells depend upon the pH of the immersion fluid. In standard solution which is rendered alkaline, the cell swells and its filopodia are transformed into large bulges which may become detached as vesicles and eventually disintegrate. Cellular aggregates break up into single cells at pH above 9.4 and below 4.2. Beyond this range,

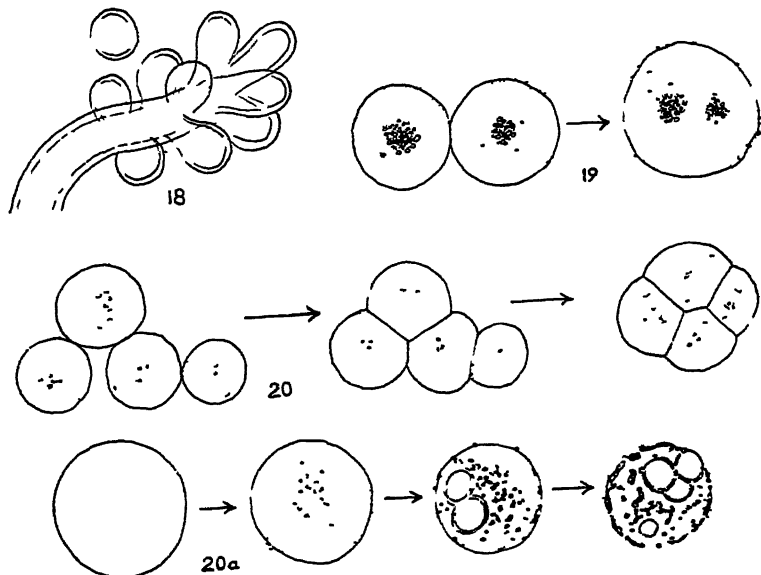


FIGURE 18 Accessory buds and fragmentation occurring in a myelin tube under the influence of alkali

FIGURE 19 Coalescence of two myelin vesicles in a 5×10^{-4} M solution of CaCl_2

FIGURE 20 Aggregation of myelin vesicles in a 10^{-2} M solution of CaCl_2

FIGURE 20a Shrinkage, granulation and collapse of a myelin vesicle under the influence of acid or other desolvating reagents

amoeboid motility ceases, and the cells eventually die. On the acid side of this range, the dead cell membrane becomes increasingly adhesive to glass or to lecithin vesicles, and the content of hyaline cell fragments precipitates into granules. Strong acids cause complete shrinkage and collapse of the fragment into a coarsely granulated film spreading on glass (FIGURE 10).

Effects of Metallic Ions. The rate of swelling of lecithin in solutions of the chlorides of Na, K, Li, Ca, and Mg varies with the salt concentration and with the valence of the cations, bivalent cations having a much stronger inhibiting effect than monovalent cations (FIGURE 17).

In molar concentrations, all chlorides completely suppressed myelin formation. Equimolar solutions of NaCl, KCl, and LiCl apparently had an identical inhibitory effect which was no longer noticeable at concentrations below 10^{-2} M, while that of CaCl_2 and MgCl_2 could still be traced at concentrations as low as 10^{-3} M. Morphologically, the effect of the various metal ions was similar to that of hydrogen ions. In higher concentrations of the bivalent cations, only small spherical myelin bodies were formed. Length and width of the bodies increased with a decrease of the salt concentration. When expanded myelin bodies were transferred from distilled water to increasingly concentrated solutions of any of the

chlorides they shrank, developed vacuolar and granular structures both within their internal fluid and in the outer membrane, and finally shrivelled up into compact bodies or, if attached to a substratum, into a reticulo-granular film (FIGURE 20a).

In accordance with its strongly dehydrating action, calcium chloride rendered myelin bodies adhesive at concentrations as low as $5 \cdot 10^{-5}$ M. When, in more concentrated solutions of CaCl_2 or MgCl_2 ($3 \cdot 10^{-4}$ M and stronger), two or more lecithin vesicles were brought into contact with each other, they either flattened one against another, forming close-packed aggregations comparable with those of living cells (FIGURE 20) or fused together into a single sphere with a complete disappearance of the separating walls (FIGURE 19). Such fusions could also be obtained with the application of acid.

In a comparable way, the rate of water uptake of living amphibian cells, or their hyaline fragments, varies markedly according to whether the culture fluid contains mono- or bivalent cations. At concentrations which cause complete dehydration of myelin bodies, all the chlorides used transform a hyaline fragment into an adhesive film studded with granules and having the appearance and basophilic properties of a collapsed lecithin vesicle. The embryonic cells attain maximal lobopod formation and amoeboid motility in relatively strong solutions of NaCl or LiCl (around $5 \cdot 10^{-2}$ M). Lower salt concentrations cause cytolysis preceded by an extreme expansion, loss of elasticity and partial dispersion of the cell membrane. In pure solutions of CaCl_2 or MgCl_2 , of any concentration, the cells cannot be kept alive for a longer period. At concentrations around $5 \cdot 10^{-2}$ M, these salts produce permanent gelation of the endoplasmic wall, slowly moving pseudopods, and finally total coagulation of the cell. Like myelin vesicles, living embryonic cells are non-adhesive in standard solution lacking calcium. The minimal amount of CaCl_2 necessary for cellular aggregation in a 0.35 per cent NaCl solution at pH 8.0 is about 10^{-3} to 10^{-6} M.

A process comparable to the coalescence of adjacent lecithin vesicles has been observed in blastula cells of *Amblystoma*. When exposed to standard solution of double strength or having a highly increased ratio of CaCl_2 , the separating walls of a number of cells disappeared and multinuclear syncytia were formed. A mutual or multiple coalescence may occur both in normal and pathological cells (fusion of gametes; syncytrophoblast of the placenta; giant cell formation in cultured tumor cells, etc.). The phenomenon seems to involve the disappearance of a layer of bound water at the interface of the cells in contact.

Several other metal compounds, such as gold chloride, mercuric chloride, silver nitrate or osmium tetroxide likewise strongly dehydrate both myelin bodies and hyaline cell fragments, while sodium oxalate and citrate influence myelinitic growth no more drastically than do corresponding concentrations of sodium chloride. It seems that the dehydrating power increases with the valence of the cation. Experiments carried out

with uranyl nitrate showed this compound to be still more effective than the bivalent cations, since its inhibitory action upon myelinic swelling could be noticed at concentrations as low as $10^{-7}M$ (FIGURE 17).

Lecithin vesicles which have become completely dehydrated through the action of heavy metal ions can no longer be reversibly hydrated and are more or less insoluble in alcohol and ether. This suggests that the well-known toxic and cytolytic action of heavy metals may arise, in part at least, from their irreversible combination with the phosphatides of the cell. According to Waugh and Schmitt (1940), copper salts at a $10^{-5}M$ concentration render the envelopes of erythrocytes largely insoluble in organic solvents and insensitive to pH and to the absence of electrolytes. It is thought that the heavy metal ions form cross-linkages with the lipids and proteins of the cell membrane, thereby disarranging the molecular pattern and increasing the permeability of this structure (see, also, Dawson and Danielli, 1938).

Palmer and Schmitt (1941) have analyzed the x-ray diffraction patterns of cephalin emulsions which had been subjected to the action of various electrolytes. $CaCl_2$ was found to be much more effective in reducing the long-period spacings of the lipid structure than were equivalent concentrations of NaCl or KCl. The pronounced desolvating effect of calcium ions was ascribed to their combination with the phosphoric acid groups of the bimolecular lipid leaflets, and the ensuing expulsion of water from between the polar interfaces of the leaflets. A similar mechanism was assumed to account for the desolvating and flocculating action of basic proteins, such as histone, upon phosphatide emulsions (Palmer, Schmitt, and Chargaff, 1941). This concept appears to give a valuable clue to the interpretation of cellular adhesiveness. Schmitt (1941) observed that when small amounts of histone or thorium salts were added to a suspension of red blood cells, the cells were tightly drawn together and formed aggregates. The phenomenon was explained as resulting from an attachment of the introduced cations to the acidic groups of the cell membrane, causing a desolvation and linking-up of the molecules in the adjacent cell surfaces.

Antagonistic Effects of Monovalent and Bivalent Cations. Prolonged exposure of lecithin to molar concentrations of $CaCl_2$ or $MgCl_2$ renders the substance incapable of swelling again in water. However, myelin vesicles which have suffered only a partial dehydration in less concentrated solutions of these salts will swell again when the solutions are diluted with water. The desolvating effect of the bivalent cations can likewise be counteracted by monovalent cations. Thus, moderately dehydrated myelin bodies which have remained unchanged in a $10^{-4}M$ solution of $CaCl_2$ for several hours will grow out into long myelin tubes when any of the chlorides of Na, K, or Li are added to the solution, provided the total salt concentration does not rise above $10^{-1}M$. This indicates that the combination of the bivalent cations with the phosphatide molecules

of the external membrane is, to a certain extent, reversible, and that the bivalent cations can be expelled and replaced by monovalent cations whose desolvating action is less pronounced. The reverse is equally possible. Furthermore, an outgrowth of new myelin tubes can be obtained when the immobilizing calcium solution is rendered more alkaline, whereas acidulation causes a further shrinkage of the bodies. These reactions may be reversed several times if the salt concentration is kept within a range corresponding to the one tolerated by living cells.

The apparent antagonistic effect of bivalent and monovalent cations upon the swelling of lecithin bodies is actually of a merely quantitative nature since, at sufficiently high concentrations, both achieve complete desolvation. In consequence of the competition of the different cations for a combination with the phosphoric acid groups, lecithin is capable of exhibiting a moderate rate of myelinitic growth in vertebrate Ringer, although the outgrowth is much reduced if the proportion of calcium ions contained in this solution is applied in the absence of the growth-promoting monovalent cations. The repressive effect of high salt concentrations upon the swelling of phosphatide vesicles may account for the absence of a contractile vacuole in many marine Protozoa, and for its disappearance when a fresh-water protozoan is transferred to sea water. As to the readiness with which cations are exchanged between living cells and their environment, reference may be made to the reviews which appeared in the *Symposium on Quantitative Biology*, volume 8 (1940).

Permeability of Myelin Bodies. Swelling and shrinkage of a lecithin vesicle would be impossible if its external wall were not readily permeable to water. Apart from water, a great variety of solutes have been observed to enter the vesicles. The criteria used were internal precipitations occurring under the influence of the permeated substance, or changes of color of the inner fluid.

It is clear from the preceding paragraphs that the different metal ions mentioned above have no difficulty in passing through the interfacial lecithin membrane, their rate of penetration being measured in seconds rather than minutes. Shrinkage and internal precipitations occur in solution of histone, egg albumen, and various amino acids (Holtfreter, 1946c). Hydrogen and hydroxyl ions may equally well enter into a myelin vesicle. This can be demonstrated by staining the vesicle in a solution containing an adequate pH indicator dye and observing the change of color occurring inside the vesicle after an acid or a base has been added to the solution. Like the histological "acid dyes" (fast green, eosin, orange G, acid fuchsin), the customary indicator dyes were found to enter the vesicles without producing noticeable morphological changes. These dyes do not precipitate with the lipids, hence are not "stored," and diffuse out again when the vesicles are returned to pure water. They act, therefore, quite differently from "basic dyes" (neutral red, Nile blue,

toluidin blue, basic fuchsin, methylene blue), which are taken up from very dilute solutions, become highly concentrated both in the wall and in the internal fluid of the shrinking myelin bodies, and cannot be washed out again because they combine with the phosphatide molecules which become dehydrated. Comparable differences of staining affinity for the two groups of dyes are observed in living cells and their vacuoles and other lipid structures. It must be added that the acidity of rancid lecithin changes the color of the permeated indicator dyes according to their pH sensitivity. The pH of the fluid within the myelin bodies used was found to be about 4.8.

The volume changes of the vesicles occurring in the different solutions discussed above cannot be satisfactorily explained by differences of osmotic pressure between the internal fluid and the external suspension medium, but are primarily caused by varying states of condensation of the limiting membrane. Strong concentrations of desolvating chemicals can be observed to cause a shrinkage of successive surface layers which slide over and peel off from the subjacent, less desolvated layers of the multimolecular wall of myelin bodies.* While contracting, the layers exert a marked pressure upon the enclosed liquid, which may escape explosively through a thinned-out and bursting portion of the wall. These observations may account for the fact that the cell membrane of living cells may burst not only in alkaline or hypotonic media, but also under the influence of histone, or basic dyes, which actually desolvate the membrane. It would appear that, analogous to the myelin vesicles, experimentally induced volume changes of embryonic cells are initiated by physico-chemical changes of the cell membrane rather than by osmotically controlled variations of turgor of the inner cytoplasm.

Models of Pseudopods and of Amoeboid Movements. When a fully extended myelin tube is dried on glass and subsequently covered with water, it sprouts out into numerous thin-walled tubules and vesicles (FIGURE 22). This indicates that through desiccation the homogeneous molecular layers of the tubular wall are disarranged and become oriented in new growth patterns when refurnished with water. Corresponding configurations are obtained when a swollen myelin vesicle is at first partly dehydrated by acid and then exposed to alkali (FIGURE 21). Again, the previously homogeneous surface membrane of the vesicle becomes differentiated into regions which vary in their readiness to expand when the conditions of swelling are restored. This results in the outgrowth of

* The occurrence of sliding and churning movements within the wall of a myelin body, or of translocations of particles attached to its surface, can be explained by the common assumption that the superimposed bimolecular lipid lamellae are separated by "lubricating" layers of water and may expand and contract independently. Such movements may be wrongly interpreted as indicating a liquid state of the entire wall. Similar translocations have been observed in particles attached to the cell surface of migrating amoebae (Schaeffer, 1920) and of dividing sea urchin eggs (Dan, Yamagita, and Sugiama, 1937), while churning movements occurred in the plasma membrane of denuded sea urchin eggs which had been touched by a glass needle (Chambers, 1938). It is still a matter of argument how many molecular lipid layers may be present in living cell membranes (Schmitt, Bear, and Ponder, 1936, 1938; Danielli, 1942). If this structure should consist of several molecular layers, then the flowing movements observed might involve a superficial layer having only paracrystalline structure and being, therefore, not in a "liquid" state.

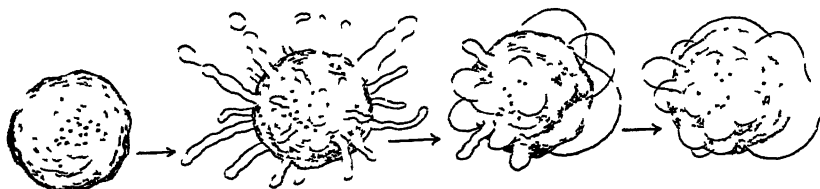


FIGURE 21 Myelin body already shrunk in acid, forms at first tubular, then bulbous processes when subsequently exposed to alkali

numerous processes, the size and shape of which resemble the hyaline pseudopods which are produced in living cells by the application of alkali (compare FIGURES 21 and 9). With a further raising of the external pH, larger areas of the surface film become expanded and the tubular processes change into voluminous bulges which, by way of local and temporal variations in the rate of solvation, constantly change their outlines. Finally, some portions of the swollen periphery may become pinched off in the form of vesicles (FIGURE 21).

Such form changes can be induced by the successive application of many other antagonistic agents which need not have more in common than that the first one has a comparatively stronger desolvating effect than the second one. Instead of using HCl or other acids, one may partly desolvate the myelin bodies by the application of bi- or multi-valent metal ions, of basic dyes, or of certain proteins (histone, egg albumen). The subsequent local swellings can be induced by the arbitrary application of alkali, of monovalent cations, or simply by diluting the solution. The process is illustrated in FIGURE 23, which shows a myelin body of the size of a gastrula cell which has first undergone shrinkage

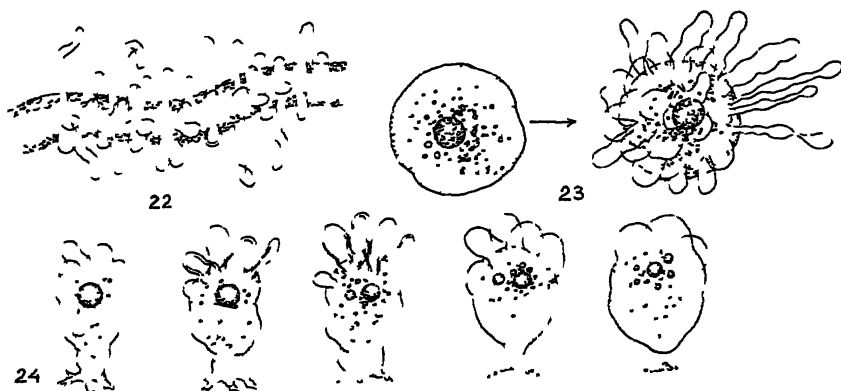


FIGURE 22 Numerous small buds growing out from a myelin tube that has been dried and subsequently wetted

FIGURE 23 Myelin body, partly dehydrated by CaCl_2 , exhibits tubular outgrowth after the addition of NaCl to the solution

FIGURE 24 Polar tubular outgrowth in a myelin body containing fatty acid droplets

through the action of CaCl_2 , and subsequently expanded into tubular and lobose processes when NaCl was added to the solution.

In addition to the external variables, local differences in the composition of the lipid body may cause the swelling process to become differentiated into a polar growth pattern. This is shown in the example of FIGURE 24, where regional differences of myelinitic activity are evidently correlated to the distribution of droplets of fatty acid which have accumulated in the upper region of the pear-shaped body. The presence of free fatty acids results from the advanced state of oxidation of the lecithin employed. While in fully expanded myelin vesicles the fatty acid particles are, in general, invisibly dispersed, any dehydrating chemical causes them to coalesce into granules and droplets of increasing diameter, which may be finally expelled into the external medium. In the present case, the myelin vesicle had been dehydrated by a strong solution of chrysoidin. The dye rendered the body adhesive to glass and stained it yellow, whereas the coalescing fat droplets stained red. Following the addition of water to the dye solution, the body performed a succession of irregular expansions and contractions, while the surface membrane in the region of the large fat droplet projected a series of pseudopodia-like processes which constantly changed their shape and eventually fused into larger bulges (FIGURE 24). With a progressive swelling of the whole body, the "foot" detached itself from the glass and the fat droplet broke up again into smaller particles which became dispersed in the internal fluid.

Differential swelling of an individual lecithin body can be obtained, furthermore, by introducing into it a particle of a dehydrating substance, such as a basic dye. When the body is immersed in water, the extent of swelling of the different regions increases with their distance from the incorporated particle. However, even in the absence of formed in-

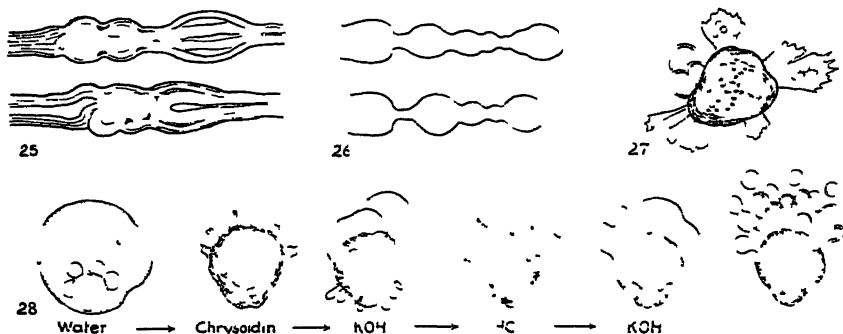


FIGURE 25 Changing pattern of varicosities in a water-immersed thick-walled myelin tube

FIGURE 26 Two phases of a constriction pattern in a myelin tube

FIGURE 27. Membranous myelin processes spreading on glass, following exposure to a strong Nile blue solution.

FIGURE 28 Alternate shrinkage and swelling of circumscribed regions of a myelin body.

clusions, myelin formations may exhibit regional differences of swelling, indicating that the walls themselves may be of an inhomogeneous composition. Frequently, a myelin tube consists of abruptly set-off segments, each having its own caliber and swelling tendency (FIGURE 25). In other instances, the wall appears to be continuous and of equal diameter, yet the body may grow out into side branches, or show along its length an alternation of bulges and constrictions resembling somewhat those observed in pseudopods (FIGURE 26). Although a regular peristaltic progression of the constrictions did not occur, it was frequently observed that terminal portions became pinched off from a myelin tube. As in living cells, fragmentation could be induced by both hydrating and dehydrating agents.

An imitation of "spontaneous" amoeboid movements was observed in the example of FIGURE 29. This myelin formation, while suspended in a dilute solution of alloxan, shrank somewhat and its surface exhibited for a longer period local bulges which were leveled out again and could reappear in the same or in another region of the body. Similar movements of a slightly periodic nature were observed in myelin formations which had been mildly dehydrated by a solution of Nile blue (Holtfreter, 1946c, Figure 16). It may be assumed that the movements were brought about by the antagonism between water and the desolvating agent, the two competing for a combination with the phosphatide molecules and replacing each other alternately.

Laminar Processes in Myelin Bodies. Fully hydrated myelin bodies which are non-adhesive to glass, to each other, and to the cell surface, will stick to and eventually spread over these surfaces when sufficiently dehydrated by any of the following agents: alcohol, acids, metal ions, basic dyes, or basic proteins. FIGURE 28 shows the successive stages of transformation produced in a myelin vesicle by the alternate application of hydrating and dehydrating agents. It will be noticed that, when the previously non-attached body shrinks and flattens over the glass surface, its periphery forms a serrated margin. In consequence of dehydration, the lipid components become arranged in new patterns, forming vacuoles, rings, and reticular ridges which can be interpreted in terms of coacervation (Bungenberg de Jong, 1932, 1935). These flowing surface patterns resemble the ruffles and networks which occur in the margin of migrating cells and, in a coarser form, in hyaline fragments that have been transformed into an adhesive film by way of dehydrating fixatives (compare FIGURES 20a, 27, 30c, and 10).

Locomotion of Lipid Bodies. It is well known that, when an oil droplet floating at the water surface is combined with a surface tension lowering substance, such as alkali or lecithin, its spreading is locally increased, resulting in the formation of mobile lobes which may become detached from the periphery. The following experiment illustrates how the local

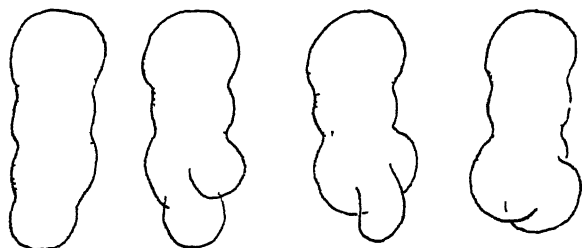


FIGURE 29 Form changes of a myelin vesicle in a solution of alloxan.



FIGURE 30 Myelin bodies showing hyaline bulges produced by alkali (a, b), or a flattened and adhesive margin with surface wrinkles, caused by acid treatment c

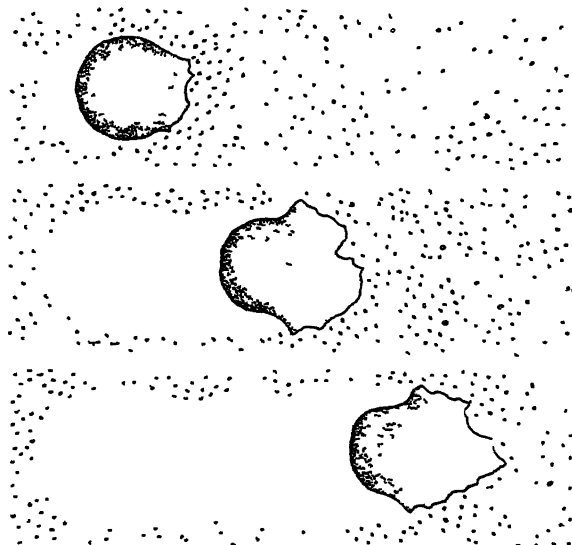


FIGURE 31 A droplet of oleic acid moving through a film of cell debris

incorporation of protoplasmic constituents may induce a unidirectional locomotion in floating oil droplets.

When an embryonic amphibian cell is brought to the air-water interface, it bursts and releases its content, which spreads as a thin film dotted with fat droplets. The latter originate from the lipochondria, which consist predominantly of neutral fat, phosphatides, and cholesterol (Fauré-Fremiet and de Streel, 1921; Holtfreter, 1946c). When subsequently a small drop of oleic acid is injected beneath this film, it attracts the adjacent lipid components of the film, which are then vigorously absorbed. That side of the drop which is incorporating the surface tension-lowering substances flattens and advances in irregular to-and-fro movements, while the opposite side remains spherical and immobile (FIGURE 31). Thus, the drop assumes the polar shape and kinetic activity of a migrating cell. It moves forward by "eating" its way through the film, leaving behind a trail free of visible particles. Eventually, the whole drop flattens out and becomes quiescent.

Membrane Formation in Relation to Gibbs-Thompson's Law. It is not claimed that the preceding experiment is an ideal illustration of the mechanism of amoeboid movements. However, it shows that the protoplasm of the amphibian cell contains substances capable of inducing surface movements when incorporated into an interfacial layer of another lipid. Previous experiments have shown that the lipid constituents of the lipochondria may combine with the dissolved protein of the yolk to form vesicles bounded by a film which has properties similar to those of a cell membrane (Holtfreter, 1947a). Apart from the granular cell inclusions, the naked cytoplasm of the amphibian egg, when exposed to any kind of hypotonic salt solution, is capable of elaborating membranous structures in the form of vacuoles or vesicles. It may be assumed that, in normal embryos, the constituents of the cell membrane are recruited from the lipoproteins of the cytoplasm and that the increase of cell surfaces occurring during development is associated with an incorporation of more of these substances into the outer membrane.

Like other surface active substances, phosphatides, obeying Gibbs-Thompson's law, tend to accumulate at interfaces. This can be demonstrated by stirring phosphatide bodies into a water-immersed drop of triolein (Holtfreter, 1946c, Figure 6). The bodies move centrifugally toward the surface of the drop, where they spread as an interfacial film showing the birefringence characteristic of radially oriented lipid molecules. It is perhaps due to the same principle that the pigment granules of the egg tend to accumulate near the egg periphery and along other interfaces, irrespective of their own specific gravity. Other possible examples of this principle are the centrifugal movements of the nuclei in centrolecithal eggs, and the migration of ectoderm cells from deeper layers into the surface of the amphibian embryo. Once the substances of various size and composition have attained a cortical position, they will react with each other and with the constituents of the external phase to form compounds and structures which are absent in deeper layers of

the system. The formation of the vitelline membrane and the external coat may be manifestations of such specific interfacial conditions. Furthermore, the pronounced spreading tendency of the ectoderm as well as the proximo-distal polarity of all cells developing from the egg surface are possibly expressions of inside-outside differentials of the egg correlated with the Gibbs-Thompson phenomenon.

In colloidal mixtures, phosphatides not only tend to accumulate at the outer interface, but they may create new interfaces in the form of internal vacuoles. The physico-chemical mechanism and the cytological implications of this phenomenon have been discussed by Bungenberg de Jong (1932, 1935). In connection with this type of coacervation, another phenomenon may be recorded. It was observed that, when the composite lipid extract from lipochondria was spread on glass as a thin layer and covered by a Nile blue solution, it became parceled up into cell-like compartments, differing one from another in their lipid composition, each unit being surrounded by a film of phosphatides (Holtfreter, 1946c). This process recalls the neoformation of cell boundaries in polynuclear syncytia, such as occurs in slime molds, or in the blastoderm of various egg types.

General Conclusions

The wide range of similarities existing between the living cell membrane and artificial phosphatide structures is a challenge to base an interpretation of the functions of the cell membrane upon some of the concepts gained from lipid models. In both the cell and myelin bodies, there was an interrelationship between the phenomena of adhesion, swelling, and motility. When embryonic cells were exposed to conditions which favor the swelling of myelin bodies (high pH, lack of calcium, low salt concentration), they underwent the following changes: loss of adhesiveness, increase of the amount of ectoplasmic fluid, rounding-up of filiform into lobose pseudopods, spreading of amoeboid motility over previously quiescent areas of the cell surface, acceleration of the movement, increase in the rate of endoplasmic eruptions. These phenomena could be reversed by the application of agents such as low pH, hypertonicity, or calcium, which produced shrinkage and adhesiveness in myelin bodies. However, prolonged exposure of the cell to the liquefying agents caused irreversible changes. Amoeboid motility faded out, the endoplasm became dispersed throughout the spherically swollen cell, the cell membrane became flabby and permeable to cell inclusions of increasing size, and finally either coagulated or disintegrated entirely.

Evidently, these phenomena of cytolysis are primarily caused by irreversible changes in the non-lipid constituents of the cell, particularly by a breakdown of protein compounds. An analogy with myelin vesicles exists only in so far as their walls likewise become thinned out and may burst when exposed to alkali. It is probably because of the comparatively

greater complexity of the cell structures than their irreversible changes occurring under extreme culture conditions find no true analogy in the behavior of lecithin vesicles. Therefore, a comparison of the two systems should be confined to the conditions which maintain cellular viability.

Polar Structure of the Cell Membrane. The kinetic behavior of isolated amphibian cells showed that their capability of assuming specific shapes develops gradually, but that, independent of their prospective significance, the neurula cells already possess an antero-posterior polarity. This primordial organization was manifested in polar differences of adhesiveness and amoeboid activity, as well as in the tendency of the cells to elongate reversibly into cylindrical bodies. While undergoing further differentiation, the individual cells acquired new and more elaborate patterns of adhesiveness and motility, which determined their direction of locomotion and their tissue-specific shapes. This process was associated with an intracellular digestion of the embryonic lipoprotein inclusions and, apparently, with a certain amount of dehydration.

Observational evidence pointed to the conclusion that the polar organization of the embryonic cell is primarily controlled by the cell membrane. The facts that the posterior pole always represents the originally distal side of the cell, and that the uncoated endoderm cells from the interior of the gastrula do not clearly reveal a polarity, seem to indicate that the polarity originates from the inside-outside gradient of the egg mentioned above. The polar characteristics are partly due to the fact that the posterior cell pole retains some of the properties of a coated cell surface, such as reduced permeability and adhesiveness, pronounced contractility, and the absence of a fluid layer between plasmagel and cell membrane.

However, these considerations do not yet explain the capability of the cell to form cylindrical or actinian-shaped bodies. This faculty must arise from axial differences of structure encompassing the whole cell surface. It has been shown above that, as a consequence of induced local differences of swelling power, the originally homogeneous surface layer of a myelin body may become heterogeneous and exhibit localized tubular growth. Accordingly, it may be assumed that, because of regional differences in the composition of the cell membrane, its response to solvating reagents increases gradually from the posterior to the anterior cell pole, and that in later stages further local differences of condensation of the membrane are developed.

In ectoderm cells, the faculty of adopting a specific shape arises at the time of their cytological determination, suggesting that the process of induction liberates certain compounds, perhaps proteins, which become integrated formative elements of the outer membrane. There is no fundamental difficulty in explaining the various forms of amoeboid movement in terms of solvation of oriented lipid layers of the cell surface. However, it is conceivable that tangentially arranged fibrillar protein

constituents of the cell membrane not only determine the elasticity of the cell surface (Harvey and Danielli, 1936) and contribute in differentiating the shape of the cell, but that in cooperation with the lipids they are directly responsible for amoeboid movements. Analogous to myosin micelles, proteins contained in the cell membrane may undergo reversible molecular contractions and extensions. The fact, however, that, in contrast to the lipid models, no protein models have as yet been devised which imitate in any way the various functions of the cell membrane, speaks in favor of the view that the essential kinetic elements of the membrane are the lipids rather than proteins.

Cellular Adhesiveness. It appears that the most important general factor determining, simultaneously, cellular adhesion, permeability, and motility is the state of solvation of the cell membrane. The physiological importance of the mono- and bivalent cations in controlling these phenomena is possibly derived from their readiness of substituting each other in protoplasmic compounds. Adhesion and aggregation of embryonic amphibian cells can be brought about by calcium ions in the ambient salt solution, provided the latter is isotonic and not too alkaline. The bivalent cations seem to operate according to the "zipper mechanism" of Schmitt (1941), by desolvating and pulling together the contacting cell surfaces. This concept presupposes a preponderance of acidic groups, possibly phosphoric acids, at the surface of the cell membrane, an assumption which finds support in the cataphoretic behavior of some cells, particularly of erythrocytes.

While in early embryonic stages only calcium and hydrogen ions seem to be required in order to hold the cells together, an intercellular adhesive matrix, presumably of a protein nature, is reported to exist in between the cells of differentiated epithelia (Gray, 1926; Chambers, 1940). Further insight into the nature of such cementing substances may be gained from investigations of the kind which Chargaff and co-workers (1944) made on the thromboplastic lipoproteins and their function in the process of blood clotting.

The cells in the interior of early amphibian embryos are not firmly aggregated. This is, perhaps, a prerequisite for the execution of the morphogenetic movements which occur during this period. The lack of cellular adhesion apparently arises from the absence, or inactivation, of agglutinating substances in the body fluid, since the cells of the different germ layers will indiscriminately unite with each other when exposed to a balanced salt solution.

With progressive differentiation, there arise cell-specific differences of adhesiveness which are reflected in the display of histotypical patterns of aggregation, disaggregation, migration, and recombination of the various cell strains (Holtfreter, 1939, 1944). It is not known whether these manifestations of a selective adhesiveness result from a molecular lock-and-key mechanism of the naked cell surfaces (Weiss, 1941) or from the

interference of specific cementing substances, which may either exudate from the contacting cells themselves or be furnished by the external fluid. Weiss (1947), in a recent discussion of the significance of cellular "affinities" and "disaffinities" for the elaboration of growth patterns, has drawn attention to the similarities which seem to exist between these phenomena and serological reactions. However, aggregations are possible between cells or myelin bodies, the membranes of which may be assumed to have an identical molecular configuration. Nor is it likely that the adhesions occurring between cells and other substances, such as glass, oil droplets, fibrin, etc., are caused by the presence of complementary sets of polar groups at the contact surfaces, comparable to those which are thought to determine antigen-antibody reactions. The ease with which cellular adhesions can be broken up and restored, and the fact that cells migrate in spite of adhesion, indicate that the bonds of attachment are very labile. When observing the gliding movements performed by the surfaces of contacting cells, one gains the impression that the attraction forces operate across an intercalated cushion of water (Holtfreter, 1946b), suggesting that the merely temporary adhesion of migrating cells may be controlled by long-distance forces of the kind which produce the tactoid formation in certain colloidal sols.

Amoeboid Movements. The general rule that cellular adhesiveness decreases with the swelling of the cell likewise applies to the different regions of the individual cell, for the surface of an expanded hyaline bulge is non-adhesive in contrast to the more contracted region of the cell. It seems, therefore, that both non-adhesiveness and expansion of the cell membrane involve a state of increased solvation of this structure, whereas a more contracted and adhesive condition of the membrane would indicate the loss of bound water. The amoeboid movements described above consist essentially of two phenomena: (1) periodic expansions and contractions of certain areas of the cell membrane; and (2) a more or less regular propagation of these alternate states of activity along the antero-posterior axis of the cell. The first phenomenon appears to be more readily accessible to an interpretation than the second one.

With Fauré-Fremiet (1929), it may be assumed that, analogous to the phosphatide models, the movements reflect periodic variations in the spacing of the radially arranged lipid molecules contained in the cell membrane. X-ray analysis and polarization-optical studies of phosphatides indicate that variations in the water content of these structures not only alter the distance between the bimolecular lipid leaflets but also affect the interchain packing of the molecules (Palmer and Schmitt, 1941). Both in cells and phosphatide models reversible expansions and contractions of the external membrane can be brought about by the alternate application of a great variety of respectively solvating and desolvating agents. However, it is a matter for conjecture what are the intracellular processes that produce the movements in the absence of ex-

ternal stimuli. Probably an indispensable regulative role in the kinetic function of the cell membrane can be ascribed to the antagonistically active hydrogen ions and bivalent metal ions on the one hand, and the hydroxyl ions and monovalent cations on the other. In addition, a reversible interaction between the phosphatide molecules and the cholesterol and proteins present in the cell membrane may determine the extent of expansion of this layer. Bear, Palmer, and Schmitt (1941) have shown that, in the presence of water, the previously incompatible molecules of various lipids (phosphatides, cerebrosides, and cholesterol) will form a homogeneous mixed phase where the different constituents become lined up side by side, showing a single identity period. Monolayers of lecithin undergo a closer molecular packing and, hence, a contraction, when cholesterol is intercalated between the lecithin molecules (Leathes, 1925). In their attempt at interpreting the structure and function of the cell membrane on the basis of coacervate models, Bungenberg de Jong and co-workers (1932, 1935) have pointed to the desolvating and tightening effect of cholesterol, triolein, and oleic acid upon phosphatide structures, this process being strongly influenced by the presence of electrolytes.

As to the effect of proteins upon the packing of oriented phosphatide layers, reference has already been made to the pronounced desolvating action of basic proteins (histone, albumen). However, since the resulting lipoprotein compounds are practically water-insoluble, it appears improbable that strongly basic proteins are engaged in amoeboid surface contraction, which is a reversible phenomenon. More adequate models of the molecular interactions in lipoprotein membranes seem to be represented by the complex monolayers studied by Schulman and Rideal (1937). These authors showed that certain mixtures of cholesterol and wheat gliadin form a composite liquid film on the air-water interface, which gels on compression. When the pressure is further increased, the film liquefies suddenly, because the gliadin is driven from the surface film into the underlying water. On decompression, the protein molecules reenter the cholesterol film, which expands. This gelation-liquefaction process is reversible several times. It is very sensitive to pH and to the ratio of the lipid-protein concentration. The applicability of these studies to cytological problems is underlined by the observation that cytolytic agents, such as fatty acids or soaps, rapidly penetrate and disperse the artificial lipoprotein films, while such agglutinating substances as tannic acid and gallic acid link the protein molecules together into a hydrophobic "skin" which resists the penetration of soaps.

On the basis of the above considerations, amoeboid movements may be conceived as resulting from localized and alternate states of solvation of the lipoprotein envelope of the cell, these changes being caused by reversible interactions between the radially arranged lipid molecules and other compounds, possibly proteins. The efficiency of this mechanism depends upon a balanced ionic atmosphere. The process may be

somewhat analogous to the reversible combinations occurring between numerous enzymes, or pigments, and their carriers, where again lipids and proteins are the most universally present constituents of the systems (Needham, 1942, p. 206; Chargaff, 1944). As to the periodicity and propagation of the surface movements, electric phenomena, comparable to those which accompany the nerve impulse, appear to be involved. Hubbard and Rothschild (1939) made the interesting observation that both the unfertilized and segmented eggs of the trout exhibit rhythmical changes of impedance, of the frequency of about 1.5 per minute. The changes are interpreted as possibly caused by a thickening or thinning of the protoplasmic membrane, involving "a change in the ability of polar or oriented molecules to rotate according to the sense of the applied current."

Summary

Based mainly upon observations on amphibian material, the attempt has been made to show that many embryological phenomena may be better understood if we take into consideration the properties and functions of the interfacial membranes which separate the cells from each other and from the external medium. While all cells are furnished with a living plasma membrane, the periphery of the amphibian egg, and the epithelia deriving from it, possess an additional covering in the form of a coat, which resembles in many ways the hyaline layer in echinoderm eggs. The coat, though not considered to be a living and indispensable part of the egg, plays an important role in determining the viability of the embryo under various environmental conditions, and in controlling, but not causing, the morphogenetic movements of gastrulation and neurulation. This structure seems to consist predominantly of protein compounds, containing calcium, which with progressive differentiation become more densified and less soluble.

The cells derived from the egg periphery possess a proximo-distal polarity which is expressed in polar differences of adhesiveness and amoeboid activity, and in the tendency of these cells to stretch themselves reversibly along an antero-posterior axis. These phenomena are thought to result from regional differences in the composition of the cell membrane, which in turn reflect an inside-outside gradient of the egg connected with the Gibbs-Thompson effect. Subsequent form changes and directed locomotions of the cells are decisively influenced by their inherent axial polarity.

The kinetic behavior of isolated embryonic cells under various environmental conditions indicates that cellular form changes and locomotion result primarily from rhythmic expansions and contractions of the cell membrane. The endoplasmic core, which is more or less separated from the outer membrane by a layer of ectoplasmic fluid, may undergo cyclic sol-gel formations which are, however, not the cause of amoeboid

movements. A cell becomes fragmented if a constriction wave passing over the cell surface becomes stationary and cuts progressively deeper into the cell body. This phenomenon is brought into relation to normal cytoplasmic division.

Local differences of cellular adhesiveness and amoeboid activity, arising in the course of development, are regarded as the main factors which transform the primitive cell into the specific shapes characteristic of the different cell strains. General features of this differentiation process are the replacement of lobose pseudopods by filiform and lamellar processes, attachment of the cell membrane to the plasmagel over the major surface area of the cell, and progressive confinement of amoeboid motility to the tips of the extended pseudopods, which remain free of endoplasm. This process seems to involve a certain amount of dehydration since, on exposure to liquefying solutions, half-way differentiated cells may readopt the appearances and kinetics of earlier developmental stages. After the cell-specific shape has been established, it may become consolidated by the elaboration of an inner cytoskeleton.

The direction of cellular migration, and the histotypical groupings and regroupings exhibited by the various types of cells in a developing organism, appear to be controlled by a selective adhesiveness of the cell membrane, which varies with the developmental stage and with the kind of cells involved. Cellular adhesiveness depends both on the chemical constitution of the contacting cell surfaces and on the composition of the immersion fluid. From the observed antagonistic effects of hydrating and dehydrating agents upon cellular adhesion, it may be concluded that the most universal and essential factor determining adhesion is the degree of solvation of the cell membrane. A prerequisite for cellular aggregation appears to be a certain concentration of hydrogen and calcium ions in the ambient solution.

The attempt is made to interpret the kinetic functions of the cell membrane on the basis of data on the chemical and physical properties of this structure. According to the evidence available, the limiting plasma film of eggs and somatic cells consists predominantly of alternating lamellae of oriented protein and lipid molecules, the latter being chiefly represented by phospholipids. In support of this concept, it is shown that many of the features pertaining to cellular permeability, adhesion, and surface movements can be imitated in models of hydrated phosphatide bodies which are subjected to various environmental conditions. The surprising similarities in the physico-chemical behavior of cells and myelin formations suggest that amoeboid movements result from alternate states of packing of the oriented lipid molecules of the cell membrane. These changes seem to involve reversible variations in the extent of solvation of the lipid leaflets, brought about by the action of electrolytes and, perhaps, proteins and other compounds. The wave-like propagation of the alternate states of film condensation along the cell surface recalls the impulses traveling along a nerve fiber. The above

concept would facilitate an understanding of the fact that cellular motility, adhesion, and permeability are usually correlated phenomena and that all three are affected when the cell is acted upon by agents which interfere with the physico-chemical conditions of the cell membrane.

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Discussion of the Paper

DR. ROBERT CHAMBERS (*New York University, New York, N. Y.*):

The study of imitations of living processes by the use of non-living material has its good points but requires caution. The plasma-membrane is a structure of which we still know practically nothing. To compare it with a lecithin-like film or even to regard it as a lipoprotein is still a questionable procedure. The plasma-membrane may extraordinarily resemble a lecithin film, but its reaction to electrolytes is quite different. Dr. Holtfreter pointed out that the presence of calcium in the medium stiffens a lecithin film and suggested that this offers still another resemblance to the cell membrane. Let us consider the sea urchin egg. The external component of the surface of the egg can be shown to be an extraneous coat which overlies the "plasma-membrane" and which is removable without detriment to the life of the cell. On the other hand, the selectively permeable and physiologically essential component of the surface is fluid in the presence of calcium and can be shown to be highly liquid when the calcium in the medium is in excess. It is the extraneous coat and not the protoplasmic surface film or the so-called plasma-membrane which is stiffened in the presence of calcium.

DR. DOUGLAS MARSLAND (*New York University, New York, N. Y.*):

I was most interested in Dr. Holtfreter's observations bearing on the physiology of amoeboid movement. However, I find it difficult to agree with his conclusion that the force of this movement originates in the membrane of the cell rather than in the plasmagel layer.

One difficulty lies in the fact that the total tension developed in the cell membrane—as measured in a variety of cells, by Harvey, Cole and others—is of a very low order, not in any case exceeding about 5 dynes per centimeter; and such a force does not seem adequate to motivate the movements of the cell. Radical deformation of the shape of the cell by so small a force would not be possible, especially at such times as the plasmagel layer is firmly set—as it is while active movements are progressing. Also, Dr. Holtfreter implies that the force that leads to cleavage likewise resides in the membrane; yet, at the time when the cleavage furrow cuts through the cell, the cortical plasmagel layer of the egg displays a maximum firmness which resists any displacement of cortical granules even by relatively high centrifugal forces.

A second difficulty is that solation and gelation are not visible processes which can be observed directly under the microscope. The hyaline protoplasm immediately subjacent to the cell membrane is capable of undergoing gelation and of developing contractile forces, although Dr. Holtfreter seems to assume that this hyaline layer is always in the state of a sol.

The cell membrane is important, no doubt, as regards the orientation of amoeboid movement, since any local diminution of the membrane ten-

sion would be conducive to the outbreak of a pseudopodium at the weakened point. Also, the firmness of attachment between the cell membrane and the subjacent plasmagel must be important, since a pseudopodium can form only in areas where detachment occurs. Such a detachment permits an outflow of plasmasol into the incipient pseudopodium. If a residual layer of plasmagel persists subjacent to the area of detachment, the outflowing sol is sieved free of granules and is perfectly hyaline. However, sometimes the "plasmagel sieve" disintegrates entirely, and then the outflowing sol is filled with granules. But whether it is hyaline or granular, the plasmasol of the pseudopodium has a capacity to gelate, and this gelation guides and supports the pseudopodium and finally limits the extension and permits the pseudopodium to undergo retraction.

The dissociated blastomeres of the amphibian embryo provide an interesting material for the study of amoeboid movement. Free-living amoebae sometimes display a similar set of unusual patterns of movement, but not as frequently or as plainly. However, to discard the sol-gel interpretation of amoeboid movement on the basis of these new observations does not seem either necessary or justifiable.

DR. WARREN H. LEWIS (*The Wistar Institute of Anatomy and Biology, Philadelphia, Pa.*):

Little has been said, so far, about the mechanics of development or the forces involved in morphogenesis. Dr. Holtfreter has considered the role of the "cell membrane" in the mechanics of cell movements. I do not agree with some of his ideas, but there is not time to consider them here, as I wish to deal especially with the mechanics of development and morphogenesis which have not been mentioned.

All eggs and cells have superficial gel layers (gel layer for short) which exert continuous contractile tension, a fundamental property of protoplasm when in the gel state. Gel layer and endoplasm are reversible states of the cytoplasm. The superficial gel layer corresponds more or less to the cell membrane of Holtfreter and to the plasmogel plus plasmolemma of the amoeba. It consists of a highly viscous surface layer that shades into somewhat less viscous gel layer. The surface layer corresponds to plasmolemma of Mast and Holtfreter's "surface coat" of amphibian eggs. It is part of the superficial gel layer and might be designated as surface coat. The superficial gel layer plays a leading role in the mechanics of development. Its contractile energy is one of the principal forces involved in morphogenesis.

Local increases and decreases of the contractile tension of the gel layer are responsible for changes of cell form, cell locomotion, flow of endoplasm, cleavage of cells, and (during development) for the cleavage of the egg into its many cells—every cell of the blastula and of even later stages has, for a time, some of the superficial gel layer of the one-celled egg carried into the depths by the contraction of an equatorial

band of the gel layer which produces the cleavage furrow. They also account for infiltration and interpenetration by migration of individual cells among others of their own and of different types and for other equally important processes.

When firmly adherent cells of epithelial membranes act together, quite different phenomena result. An increase of the contractile tension on one surface of an area of adherent cells that resist distortion will result in a concave depression, or invagination, on the side of the greater tension. Invagination plays an essential role in early development of blastopore, neural tube, optic vesicle, optic cup, lens, otic vesicle, nasal pit, and probably all other organs that arise from epithelial membranes and tubes.

In addition to cell migrations and invaginations, pulls, pushes, and squeezes produced by contractions of gel layers play essential roles in development.

The zebra fish egg has a strong gel layer that exerts contractile tension in all tangential directions. Contraction of the yolk part squeezes endoplasm out of the yolk to form the disk as its part of the gel layer relaxes. Large yolk globules are held back by a sieve zone. After endoplasm is squeezed out of the yolk, its globules are compressed into polyhedrons by continued contraction of yolk gel layer. During gastrulation, contraction of the yolk part of the gel layer pulls the attached blastodisk over the yolk and at the same time thrusts (pushes) the latter against the disk. As yolk gel layer contracts, it solates and by the time disk edge is pulled to the vegetal pole it has entirely solated. Inner active disk cells migrate around its edge to produce involuting germ ring.

All surface cells of *Amblystoma* blastulae are strongly adherent to one another by their superficial gel layers. The presumptive areas, vegetal pole, dorsal entoderm, ventral entoderm, mesoblast, chordablast, neuroblast, and ectoblast behave differently.

Most vegetal pole area cells migrate inward away from the surface. Some invaginate with adjacent dorsal entoderm at the blastopore and produce a shallow archenteron. As the superficial ends of vegetal pole and dorsal entoderm cells contract, they pull adjacent adherent ventral entoderm toward vegetal pole and blastopore and chordablast to the edge of the dorsal lip. These, in turn, pull mesoblast and ectoneuroblast toward the vegetal pole and blastopore.

The arched dorsal lip contracts and advances and pulls row after row of chordablast cells over its edge against ventral entoderm, like the treads of a caterpillar tractor turning to the ground. This elongates chordablast, extends archenteron posteriorly, covers endoderm (yolk plug), and reduces blastopore size. As chordablast is turned under, it pulls adherent neuroblast toward and finally to the blastopore edge. Neuroblast is elongated and narrowed by this pull.

In the meantime, the superficial ends of the ventral entoderm cells contract and reduce its area. This pulls adherent mesoblast and ecto-

blast toward the blastopore. The superficial ends of mesoblast cells at the mesoentoderm line contract and the cells migrate inward. This pulls neighboring mesoblast cells to this line where they, in turn, migrate inward until all leave the surface. This pulls adherent ectoblast to the blastopore. Mesoderm ingression and dorsal lip contraction result in a contrasting constriction around the mesoderm-dorsal lip circle which is partly responsible for dorsal lip advance and blastopore closure.

These vegetal hemisphere contractions reduce its area and push or thrust yolk mass against blastocoele and ectoneuroblast and help to stretch the latter as it is pulled toward the blastopore.

Archenteron expansion is probably produced by pressure of fluid secreted into it by its lining cells. Secretion of fluid plays an active mechanical part in the development of blastocoele, mammalian blastocyst, brain ventricles, optic cup, ear vesicle, etc.

The factors responsible for the superficial gelation of the cytoplasm and for local changes of its contractile tension are unknown.

DR. J. HOLTFRETER:

In reply to Dr. Chambers, I should like to refer to what I said with regard to the difficulty of determining by microscopic observation, whether or not such a delicate structure as the plasma membrane has the properties of a liquid (see footnote, p. 739). Apparently, all workers who have tried to elucidate the physico-chemical properties of this structure agree that the outermost layer of living cells is organized in such a way that its molecular constituents cannot move at random, as in a liquid phase. If the concept of a paracrystalline lipoprotein nature of the plasma membrane be accepted, then churning or other movements observed at the surface of denuded eggs should be due to variations in the molecular packing of the organized surface layer. Furthermore, analogous to the translations occurring within the wall of a myelin body, "flowing" motions in the cell surface might be caused by the slipping of a molecular surface lamella over a subjacent lamella. As far as the embryonic amphibian cells are concerned, their living outer membrane appears to have normally a semi-solid consistency. Without the support of the endoplasmic gel wall, it can spontaneously exhibit folds of elastic tension, mobile ruffles, and long tubular or attenuated pseudopodia. This seems to indicate that the membrane is not fluid, and that other forces than surface tension determine its shape and motility. However, prolonged exposure to salt solutions lacking calcium renders the membrane flabby, non-contractile, and highly permeable, whereas, in solutions of a sufficiently high calcium concentration, the membrane shrinks into a definitely solid substance which resists being cut with a glass needle.

With reference to the remarks of Dr. Marsland, I should like to point out that the extraordinary size of embryonic amphibian cells makes it easy to observe their protoplasmic architecture under the microscope. In fact, both the structural and kinetic details in these cells are so spec-

tacular that my undergraduates in embryology have no difficulty in seeing them. Using the well-recognized criterion of the presence or absence of unrestricted Brownian movement, one can readily decide whether the granulated endoplasm is in a sol or gel state. I agree, however, with Dr. Marsland that in more highly differentiated cells, when granulation diminishes and the plasmagel becomes more firmly associated with the cell membrane, these two layers can no longer be optically differentiated, except at the tip of a pseudopod, where they remain separated by a hyaline layer referred to in my paper as "ectoplasmic fluid." Since both this fluid and the plasmagel may be hyaline, their optical appearance is, of course, no criterion for the physical state of the protoplasm.

In the comparatively minute cells isolated from mammals, even such an experienced microscopist as Dr. W. H. Lewis (1942) has not always been able to recognize a gelated cortex, nor could he detect a reversible sol-gel formation when these cells were migrating. Such material is, therefore, unfavorable for an analysis of amoeboid movement. On the other hand, Dr. Lewis has published beautiful microphotographs of the advancing tip of a slime mold (1942, Figures 1-4) which show clearly that in this organism, as in *Amoeba* and amphibian cells, the frontal wall of the plasmagel is separated from the outer membrane by a spacious layer of a hyaline substance, said to be fluid. Furthermore, the outer membrane can be seen to perform lively amoeboid movements while the subjacent plasmagel wall shows a smooth surface, a fact which obviously does not fit into the sol-gel interpretation held by Dr. Lewis and others.

The various patterns of movement occurring in isolated amphibian cells of different age and tissue derivation are not unusual, since they comprise most types of amoeboid movement observed in protozoa and somatic cells. Whether the latter can be interpreted along the lines here suggested remains to be investigated. At any rate, the current sol-gel theory fails consistently to explain the various kinetic phenomena here described, whereas all these phenomena are readily covered by the membrane theory advanced as an alternative. Apparently, the mechanism here proposed is likewise applicable to the interpretation of ciliary movements.

Lack of space prevents me from discussing the ideas of Dr. Lewis on morphogenetic movements. To avoid misunderstandings, I only wish to restate my conclusion that the protoplasmic structures defined respectively as coat, cell membrane (plasmalemma), and plasmagel, differ from each other structurally as well as functionally. In cases where these layers cannot be distinguished from each other, one should not ascribe arbitrarily any function to any one of them which they probably do not perform. I am not aware of experimental data showing that the coat, or any other superficial "gel layer," has essentially more to do with the mechanics of gastrulation than has been mentioned in my foregoing account.

PROSPECTIVE AREAS AND DIFFERENTIATION POTENCIES IN THE CHICK BLASTODERM

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EMBRYOLOGISTS interested in the chick, like everyone else in the field, find an expanding technology opening up before them. In the last decade, even during the war, microchemical, histochemical, and cytochemical tools have emerged and have developed to a degree that makes it immediately possible to obtain accurate knowledge of even the earliest events in development. It is imperative for embryologists to make certain of the definition of their morphogenetic problems, in order that the new tools be applied precisely and profitably. The present opportunity to examine the factual basis of some classical concepts as applied to the chick is thus very welcome.

Germinal Movements during Gastrulation. The discussion will center around problems of the nervous system, commencing with the relationships of the medullary area to the underlying axial mesoderm. It is characteristic of our uncertainty of the course of early embryonic movements in the chick that Dr. Spratt of Johns Hopkins should have been able, just recently, to make us readjust all our ideas of early localization by his marking experiments on the uncubated blastoderm.

It will be well to recall that gastrulation in the chick is performed in at least two steps, perhaps three. The first, which has progressed to a variable degree at the time of laying, is the delamination of the lower layer or hypoblast, from an originally single blastodermal sheet. This step takes place without much superficial morphological indication. The hypoblast customarily is believed to give rise to the entoderm. The mesoderm is invaginated from the upper layer mainly by migration through a morphologically patent blastopore, the primitive streak, which takes shape in correlation with other visible changes in the embryonic area. Descriptions are available of some mesoderm, in early stages, rather delaminating irregularly from the upper layer than invaginating in orderly fashion through the streak. The first appearance of the streak itself presents this picture, which might be considered a preliminary or accessory manner of mesoderm formation.

In view of the current interest in the association of carbohydrate metabolism with invagination, it is surprising that Jacobson (1938) has thus far been the only investigator to describe the invagination or delamination process in the chick as being accompanied by loss of glycogen

in the cells involved. This would make the situation comparable to that in the amphibian gastrula, and thus far seems to indicate the only qualitative chemical difference that can be correlated with mesoderm formation.

The primitive streak arises by movement of the epiblast layer to a median posterior line; material is piled up on either side of this line; eventually, a mesodermal sheet grows out underneath, spreading at first laterally and posteriorly. Only after the streak has reached its maximum size does an appreciable anterior migration of continuous mesoderm take place, *i.e.*, the head-process with its paraxial mesoderm. This sheet is preceded by some looser, less coherent mesoderm, the "anterior free mesoderm" or, essentially, the prechordal plate, which has been briefly described by Pasteels (1936) as invaginating also through the anterior streak, ahead of the chorda.

Spratt's (1946) recent study shows with great exactness the movement of carbon marks placed on the surface of the unincubated blastoderm cultivated *in vitro*. His Figure 13 summarizes the information in a coherent diagram: it is clear that the whole posterior half of the unincubated blastoderm eventually becomes mesoderm or streak, whereas the posterior border of the anterior half is the site of a graded but—in peripheral regions—very marked stretching or expansion, considered to be an active rather than a passive process. It would seem that we have at last a definitive answer to the question of how much epiblast material goes into the streak—a question that has received widely differing answers, not only from nineteenth-century workers but from recent experimental workers as well (Wetzel, 1929; Pasteels, 1936). The fact that the work of Spratt was done *in vitro* may perhaps invite objection, but the author controlled his observations by careful measurements, comparing his preparations with normally growing ones, and by many repetitions. If, working in this artificial environment, he had found a less energetic or less extensive invagination than had previously been described, some reservation of judgment might perhaps be justified, since explantation notoriously retards or inhibits many processes. As the case is, however, one feels sure that the displacements are at least as radical as Spratt indicates.

Moog (1944) briefly describes gradients in phosphatase (especially in acid phosphatase) in early blastoderms that might possibly coincide with this concentration of material in the streak. It would be of particular interest to follow the history of regions that evidently expand most actively during this period, to determine if they are the site of enzymatic loss, as might be suggested by her findings.

The disposition of areas of the future embryo in the stage of the maximum or definitive primitive streak is well known from the maps of Wetzel and Pasteels. This plan may be re-projected back on the unincubated blastoderm, with results somewhat as in FIGURE 1. In this figure, the right half shows merely the boundaries of the streak at its inception,

and its definitive stage when the primitive pit, surrounded by Hensen's Node, occupies a position immediately behind the stationary midpoint of the blastoderm. The limit of invagination in post-definitive streak

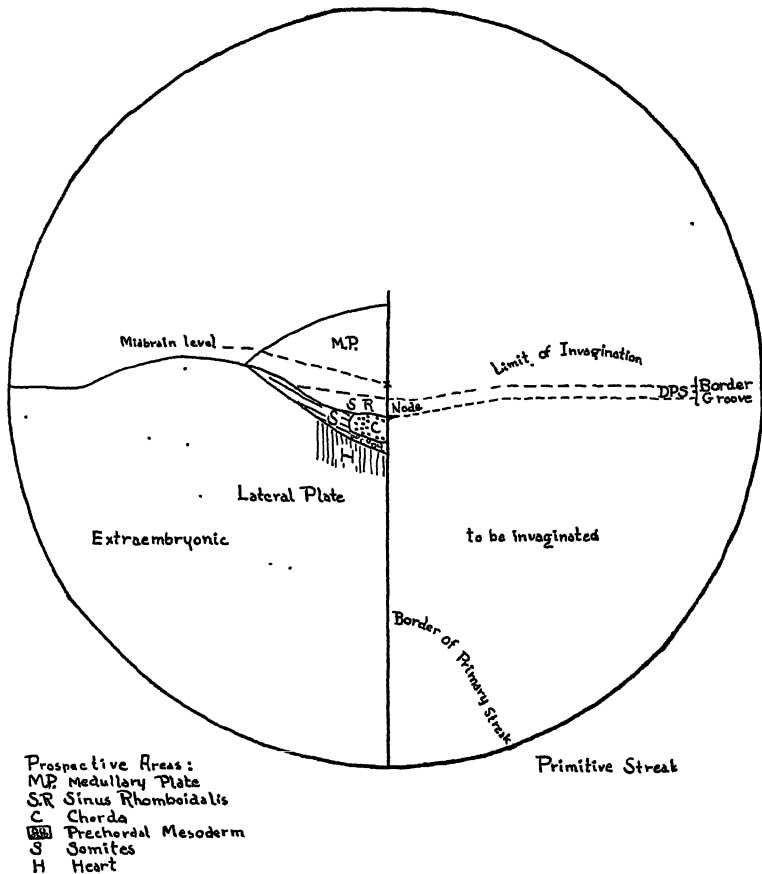


FIGURE 1. Diagram of prospective areas in the unincubated blastoderm, in the light of recent experimental results. Right side shows the primitive streak material, and limits of invagination. Left shows prospective embryonic areas. Intramesodermal boundaries are all hypothetical.

stages has also been estimated from Spratt's 1947 figures. The left side of the drawing attempts to show the organ-forming areas, on the basis of this information. The medullary area must lie anterior to the invaginating region, and the mesodermal areas must enter the streak in inverse order as compared to their final position. There is no accurate experimental basis for boundaries between the mesodermal areas, except possibly in the case of the chorda.

The line between the mesodermal area and the medullary region has

a clear basis in Spratt's records. During formation of the streak, the shape of the medullary area must change from something like a half-circle to a long ovoid, largely through stretching and wheeling of the latero-posterior borders. As the head-process grows, the anterior half of the oval will elongate through differential growth greatest in the mid-line. This process moves Hensen's Node and the primitive streak relatively posterior, and swings segment after segment into rectilinear transverse position. At the stage of the definitive primitive streak, all of the medullary plate that is really laid out, as a flat area, consists of the anterior and lateral parts. The median material, or neural floor, is still to be proliferated from the anterior border of Hensen's Node, along with the notochord.

Thus, different parts of the prospective medullary tube have definitely different histories. Some are older than others, as epithelial areas. The significance of this is evident from the consideration that the impetus toward differentiation of the medullary plate is, in all probability, given by the emigrating mesoderm that comes to underlie the ectodermal layer. The pattern of emigration of mesoderm from the streak is not known as precisely as is the history of the superficial layer, but the general lines can be sketched from morphological reasoning and from the vital stain experiments of Pasteels. The most anterior part of the medullary area is never underlain by more than diffuse prechordal material. The lateral and posterior parts, on the contrary, are underlain by a succession of mesodermal areas, as the sheet migrates laterally: first, probably, extra-embryonic blood-forming mesoderm; then heart and lateral plate; only later parachordal or somite mesoderm takes its definitive station. We definitely do not know the rate or exact directions of this migration, but its occurrence is beyond doubt. As for the neural floor, it seems to be evolved from the front of Hensen's Node *pari passu* with the underlying chorda. The two adhere closely.

Induction of Medullary Plate. The next consideration is a brief review of the evidence, for the chick, that medullary plate arises as a result of induction from an underlying layer. It will be recalled that the original proof of the dependence of the amphibian medullary plate on a stimulus from the underlying archenteron roof consisted broadly of two types of experiment: (1) the test of the ability of carefully specified germinal regions (blastopore lip, archenteron roof or later derivatives) to induce medullary plate in ectoderm that would normally differentiate into something else; and (2) the test of the differentiation capacity of isolated ectoderm, before and after it is underlain by inducing mesoderm. The second test was carried out by various isolation or transplantation methods, culminating in the systematic area-by-area investigation reported by Holtfreter in 1936.

For the chick, Waddington (1930 *et seq.*) has shown that the primitive streak and, to some extent, its derivatives have the capacity to in-

duce supernumerary medullary plate in ectoderm of widely varying regions of the blastoderm. Woodside (1937) has made clear at what age it is possible to elicit this reaction, *viz.*, response is maximal if the graft has an opportunity to act while the host streak is developing, and virtually disappears when the host is forming neural folds, lingering longer in anterior regions of the blastoderm than in posterior. It is not easy to verify, from published accounts and figures, if every part of the non-medullary ectoderm has been so tested and has proved capable of forming medullary plate when properly stimulated. Certainly, lateral parts of the *area pellucida* ectoderm and some—presumably all—of that of the *area opaca* can so respond. There seems to have been no critical test of the *area pellucida* ectoderm just anterior to the normal axis, a region peculiar in that, normally, it is never underlain by mesoderm. This test would be of interest. Nevertheless, it seems safe to say that all ectoderm of suitable age can be induced to form medullary plate and to undergo some regional differentiation.

As to the efficient stimulus, the very early streak seems not to have been tested for inducing capacity; but different parts of later streaks, and of the head-process with overlying ectoderm, have proved effective. The complexity of the inductions produced by these non-homogenous transplants acting in the growth field of a host embryo *in vitro* is extreme. This is perhaps clearest in the long series of cases reported by Waddington and Schmidt (1933), which were also heteroplastic (duck to chick and *vice versa*). In this report, the cases in which unmistakable regional character or even polarity can be ascertained in the induced medullary plate are relatively few. From these few cases, however, the authors have shown some evidence for differences in character of the induction, depending on the region (anterior or mid-streak, head-process, etc.) from which the graft was taken.

There is, thus, satisfactory evidence that medullary induction can occur in the chick ectoderm. We may proceed to the evidence that this process occurs normally. For the chick, in the stages when ectoderm is being underlain by mesoderm, separation of the two layers for experimental tests has not been technically possible. The critical, direct test of differentiating capacity of the same region of ectoderm, before and shortly after mesodermal contact, has not been performed. Only indirect approaches can be cited and they have led to new questions rather than to satisfactory answers.

Some time ago (Rudnick, 1938a), separate pieces of the early streak blastoderm were grown *in vitro*. In the earliest series, cuts were calculated—by the information then available but quite coinciding with Spratt's new data—to separate the forming streak, visibly invaginating, from superficial mesoderm about to be invaginated, and these in turn from ectoderm. No embryonic axis differentiated from any piece, but merely little clumps or vesicles of tissue. Few tissues could form after this drastic partition, namely, generalized tissues like blood, mesen-

chyme, epithelium, and, in addition, heart muscle and medullary plate. Medullary plate, or rather little tubes or spheres, developed from the most anterior (ectodermal) explants in a certain percentage of cases. It is not certain whether they did so from other pieces. At this stage, before the groove of the streak had appeared, it seems impossible that mesoderm invaginated through the streak region could have reached the anterior piece, which, however, contained all or most of the prospective medullary material. In face of this, one is reduced to two radically different alternative hypotheses: either the prospective medullary region, *in toto* or in part, contains potencies for medullary differentiation antedating mesodermal induction; or, in pieces cut and explanted, there is some irregular invagination or substitute therefor, and this process is adequate for induction. Further along this second line of thinking, there is the question of the normal prechordal mesoderm—whether perhaps its regular route of invagination is not by irregular delamination, which could have occurred in the isolated explants; in which case the medullary tubes would represent forebrain.

In this material, there was no indication as to what region or level of the axis the medullary nodules might represent. Even in explants grown from later streaks, from regions where induction by emigrating mesoderm was possible, no regional differences could be ascertained. Clearly such fragments present drastic mechanical opposition to regional morphogenesis.

Spratt (1940, 1942), a short time later, reported experiments in which regional organization had been given a much better chance. These consisted in dividing early blastoderms (pre-streak through head-process or later) into only two parts by a transverse cut. If the cut was made approximately through the midpoint of the blastoderm, in pre-streak or early streak stages, it is obvious that all or part of the prospective medullary plate would be isolated from the intact mesoderm-streak region behind. From cultivation of both pieces, Spratt obtained typically a fairly good axis from the posterior piece in which the streak formed, and a little nodule of medullary plate in the center of the anterior piece. At the time, Spratt interpreted the rather shapeless anterior bits as forebrain, and, in a few cases, on subculturing, obtained retinal pigment from them; the posterior axes he thought specifically deficient in forebrain. One wonders if, in view of his recent localization experiments, he might not wish to revise this view and think of the posterior axes as being inhibited but not necessarily lacking in any one level. It is clear that not only the prospective forebrain but a good bit besides was included in the anterior piece, whereas very little destined medullary material was present in the posterior piece. In development *in vitro*, the anterior cut border of the posterior piece (where lay most of the prospective medullary material that was included) remained fixed to the clot. This emphasizes the conclusion that the medullary plate of the posterior axes must have formed largely from epithelium that normally would

have entered the streak to become mesoderm. It would seem that Spratt has shown here, among other things, that prospective mesoderm also can be induced to form medullary plate.

The anterior pieces again present the problem of whether and by what the medullary nodules were induced. To illustrate the difference in result, when essentially the same transection experiment is performed after the whole brain-forming region is underlain by mesoderm, one should consult the figures in Spratt, 1940 (Plate 2). In these cases, the anterior piece clearly complements the posterior, and the mosaic character of the separation of forebrain or its parts from the rest of the axis is obvious.

Thus, attempts to test parts of the medullary field before formation of the mesoderm have served to demonstrate our ignorance of the normal course of invagination, not to mention our ignorance of the organizing substance itself. If, at first glance, things look as if the early medullary field in the chick (the site of the later forebrain) possesses some powers of self-differentiation, it is not likely that such an explanation will satisfy investigators or be allowed to remain without further analysis.

Regional Organization of the Medullary Plate. The forebrain problem raised in the preceding section may serve to introduce some discussion of the pattern present in the early medullary plate. In searching the literature for cases where a forebrain has been experimentally induced, one finds exactly one instance where an indubitable prosencephalon with optic swellings is figured, as contrasted with vague designations of "head" or "head-fold" which usually look very unlike any part of a normal chick brain. This case is shown in Waddington and Schmidt, 1933, Figure 10, and the beautiful induced head lies beside the normal one at about the same antero-posterior level. The transplant performing the induction was from a chick: the anterior half of a medium (*i.e.*, pre-definitive) primitive streak, including Hensen's Node (whether any material anterior to the node was also included, is not stated), placed ventral side up in the right middle region of the duck host, beneath the epiblast. If anything, the induced brain is farther advanced than the host, that is, optic swellings are clear. The transplant itself has differentiated into neural tube and a little unspecified mesoderm. The graft material immediately touching the induced optic swelling is neural tube, which is actually continuous with the host induction. The authors also note that the most anterior part of the induction is not underlain by any graft at all. It seems questionable as to whether the regional character of this induction is to be attributed, as the authors do, entirely to the character of the graft. Its position with relation to the host embryo is too parallel. If forebrain is induced by graft neural tube, as the morphological relations appear to indicate, one would think that the regional character of the induction must be attributable, at least in part, to host influences. Unless pre-nodal material were included in the graft, the normal in-

ductor for telencephalon was not present. The evidence offered by this case for localization within the inducing system is thus very unsatisfactory.

In the head-process blastoderm, each level of the medullary plate is capable of differentiating into a characteristic brain-vesicle, with accurate histological pattern of cell types and layers, when suitably transplanted, along with underlying mesoderm (Rawles, 1936). This antero-posterior pattern also seems to be present in the definitive primitive streak stage, if allowance is made for the condensed nature of the material. In preceding stages, especially in those where it is known that the definitive relations of axial mesoderm to medullary ectoderm have not been attained, no experimental evidence is available. With our current increase of understanding of the history of movements and relative position of the two layers, a real experimental test of their separate roles in the emergence of the antero-posterior pattern will be possible. The evidence found by Spratt, of potency of at least a cellular order to form retinal pigment in anterior ectodermal pieces isolated from the rest of the axis in pre-streak stages (1942), is a suggestion that the idea of pattern in the ectoderm itself must not be neglected.

Transverse organization of the medullary plate, which changes to dorso-ventral order in the embryo, is a process completed, in the cases studied, later than that of the antero-posterior axis. The cases studied are those of the eye (Clarke, 1936) and the neural crest as indicated by pigment cells (Rawles, 1940; Ris, 1941). In both instances, the histological potency to form these lateral or dorsal structures is retained by the isolated median or ventral strip some time after the formation of medullary folds. The exact movements of material in these cases have not been followed. There is a slight chance, for the eye, that a tenuous strip median in the anterior medullary plate, too narrow to be tested by current methods, may, from the first, be unable to form eye tissue, and that the diencephalic floor arises by enlargement of this strip. The situation looks much more like a gradual separation of a continuous eye field into two laterally placed ones by the actual loss of ability of the medial cells to perform a certain histogenetic task. The case of the neural crest melanophores is clearly of this type. This bilateral or dorso-ventral pattern stabilization seems to be a much slower process than medullary induction *per se*. In the amphibian forebrain, the mesoderm has been shown to play a critical role. This would certainly require some localization of pattern in the mesoderm, even if its expression in the ectoderm emerges only slowly.

A morphological basis for a medio-lateral pattern in the invaginated mesoderm is more immediately clear than it is for an antero-posterior one. It has already been pointed out that the lateral parts of the prospective medullary plate are underlain by a progressively migrating mesodermal sheet and are, thus, subject to contact with a succession of mesodermal areas. In the midline, ectoderm and mesoderm are at first massed,

without epithelial relations, in Hensen's Node, and grow forward together from that position. This process has been compared to the growth of a tailbud blastema. It is probable that intermediate gradations between these differing processes occur in intermediate regions. Thus, with reference to time and pattern of contact between the two layers, a medio-lateral differential is ready for analysis by more refined transplantation and by cytochemical methods. Furthermore, at the time these patterns are being realized, excised areas can be regenerated from surrounding tissue—even the whole of Hensen's Node (Waddington, 1930 *et seq.*). This offers another means to study the time required for acquisition of new potencies or for the loss of others. These suggestions are made in order to emphasize that, in spite of some of its drawbacks as experimental material in early stages, the chick embryo has definite morphological features which, once understood, promise to make it especially valuable for study of some general problems of vertebrate pattern.

Differentiation Mechanisms. It would seem convenient to distinguish three types of activity concerned in the differentiation of a nervous system from a medullary plate: (1) secretory, responsible for the maintenance of the various cavities of the central nervous system; (2) mass-movements within the whole epithelium, or large groups of its cells, responsible in the first place for closure and shaping of the neural tube, subsequently for formation of layers, cell columns, nuclei, etc., within definite parts of the medullary tube; and (3) differentiation of individual cells in various directions, such as neurons of several types, supporting elements, ependyma, etc. The first activity has not been the subject of adequate study, though the chick embryo would seem to offer an excellent object in which the nature of the barrier between tissues and cavities could be investigated. It is proposed to consider the second and third processes here, briefly, with reference to possible relations one to another.

The induction reaction itself is, of course, of the nature of an epithelial reaction, first visible in the marked heightening of the cells of the induced region. Subsequent processes are also related to the properties of an epithelium, not to single cells; *e.g.*, the changes in form and polarization responsible for the closure of the neural tube. Hobson's (1941) observation that closure, previous to actual fusion of the medullary folds, can be reversed neatly by dehydrating agents such as a drop of glycerine, calls for further analysis. In later stages, the formation of cell layers, so characteristic of various parts of the brain and neural tube, must also be regarded as a series of mass patterns within an epithelium.

As a contrast, we have the cellular differentiation that has been long and closely studied, whereby single cells apparently undergo the characteristic changes involved in forming neurofibrillae and putting forth nerve processes which then acquire peripheral connections. Barron's (1946) recent interpretative study of differentiation in the motor area

of the brachial region of the spinal cord may be cited. He finds that certain individual cells of the neural epithelium of the basal plate start differentiation *in situ*, but that this process is correlated with a peripheral migration to the position which will be occupied by the ventral horn. The first pioneer fibers of the ventral root come from such *primary neuroblasts*. In addition, other cells migrate out from the epithelium in an apparently undifferentiated condition, and later become *secondary neuroblasts* by differentiation in striking proximity to the dendritic processes or cell bodies of the primary neuroblasts. Hamburger and his students (*cf.* Hamburger and Keefe, 1944) have already formulated the idea of a cell-to-cell induction, which Barron supports on morphological grounds, as a result of their experimental studies on altering the peripheral load of the brachial or lumbosacral region of the cord. They have shown that the marked development of the motor columns in these two regions is directly dependent on the presence of a periphery with which the motor fibers leaving the cord may connect, and not on any central connections outside of the few segments comprising the limb area of the cord (Hamburger, 1946). Furthermore, the periphery controls the development of the motor columns not by regulating cell divisions or number of cells within the cord, but by controlling cell differentiation, *i.e.*, the formation of motor neuroblasts in the ventral horns. A cell-to-cell mechanism, whereby a pioneer neuron, once it connects with the periphery, puts out one or more dendrites and simultaneously becomes capable of inducing neighboring undifferentiated cells to follow its lead, until the periphery is loaded, provides a most attractive hypothesis, inviting direct experimental investigation. The only lack is a suggestion of what initiates the process in the first place.

The polarization microscope studies of Hobson (1941) form a very interesting beginning of optical studies of the neural epithelium just before and after closure of the tube. The indication is of an orientation of protein micells in the long axes of the cells (that is, radiating out from the center of the tube) and lipoid components perpendicular to the protein. A great share of the birefringence was observed in the various membranes (limiting, cell, and nuclear). This particular type of orientation is by no means peculiar to neural epithelium in the chick embryo. It would, however, be most interesting to make similar observations in slightly later stages, when there is more zonation in the neural wall and the pattern of cellular emigration that has just been discussed is better established. It might, thus, be possible to detect differences in content or structure of the migrating individual cells.

Moog's (1943) studies of the phosphatase distribution at various stages of the developing spinal cord show some striking patterns, some of which, such as the median band of alkaline phosphatase, seem very difficult to understand. The preparations are not figured or described minutely enough to ascertain differences of individual cells, so that it is impossible to say if phosphatase gives any promise of being involved in the differen-

tiation under discussion. The program will evidently be to continue to look for cytochemical and cytophysical differences between individual cells in the neural tube, until the specific difference between migrating and non-migrating individuals becomes apparent, as well as subsequent differences between neuroblasts and undifferentiated cells. A cytochemical cell lineage of the elements and derivatives of the neural epithelium is a highly desirable goal which, when available, will perhaps offer the clue to the mechanism or mechanisms involved.

It is possible that the two major activities in neuron formation—emigration and putting forth nerve processes—are merely steps in one single course of surface changes. Under this view, a series of events would be early initiated in all cells of the neural epithelium, which would progress more rapidly in some cells than in others. In cells changing most rapidly, some double threshold would be crossed and those cells would become primary neuroblasts, simultaneously undergoing internal rearrangement, changing external form, and losing contact with their neighbors to slip outside the epithelium. In cells where the process moves somewhat less rapidly, only an intermediate threshold would be attained. Surface changes would go only so far as to permit these cells to migrate out of the epithelium in a generally rounded state, requiring a further stimulus from outside—an induction—to become neuroblasts; these would be the secondary type. Large groups of cells in which either threshold was reached at the same time would provide a basis for cell layers, columns, etc., as in the differentiating brain epithelium.

It would be well to note that these formal relations within the epithelial structure of the neural tube do not hold any exclusive key to differentiation of neurons. Nerve cells may differentiate in transplanted material that has never had an opportunity to form a tube or more than a most irregular and distorted epithelium (Rudnick, 1938b). It is only necessary to consider the neural crest to realize that no epithelial history at all is requisite to neuron differentiation, however important it may be for the larger pattern of the central nervous system.

If it seems that the foregoing review has been more concerned with exposing our ignorance than with recording progress in the understanding of developmental processes in the chick, it can certainly be replied that the morphological task before us is a most exacting one. We are really faced with ascertaining individual cell differences and cellular relations in material in which, until recently, one cell seemed to look just like another. The fact that all cells in the blastoderm or in the neural tube do not behave alike has led to various hypotheses concerning the mechanisms responsible, *viz.*, progressive internal change; cell contact with chemical or physical effect; even production of substances and their transfer over some distance. The sorting-out of these possibilities inevitably requires more and more detailed knowledge of cellular relations in normal development. The morphological questions we are now trying

to answer would have been meaningless in the classical period of descriptive morphology, and, in this sense, it can be submitted that progress is being made.

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BIOCHEMICAL DIFFERENTIATION DURING AMPHIBIAN DEVELOPMENT*

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Studies on the Primary Organizer

BIOCHEMICAL studies of the so-called primary organizer or neural inductor of the amphibian embryo have been directed along two main lines. In the first place, following the discovery that induction of the nervous system could be brought about by agencies other than living embryonic tissues, attempts were made to determine, by various implantation techniques, the nature of the chemical substance or substances which presumably were involved in the process. The literature on this subject is not without interest, but, it must be confessed, our knowledge of the chemistry of the natural inductors has not been greatly enhanced by the numerous studies of this type. Nevertheless, it can hardly be doubted that the interaction between the invaginated chorda mesoderm and the presumptive neural plate, leading to the histogenetic and morphogenetic differentiation of the nervous system, involves the operation of specific chemical substances even though their precise nature and the means through which they exert their influence are unknown. Moreover, it seems increasingly clear, especially in view of Holtfreter's (1944, 1945) recent results, that embryonic tissue contains inducing substances in bound or inactive form and that, as suggested by Needham (1942), a number of physical and chemical agents are effective in bringing about their release.

Respiratory Rates of Dorsal Lip and Ventral Ectoderm. The second line of work has been concerned with an analysis of the metabolic characteristics of inducing and non-inducing parts of the embryo in an attempt to determine whether the developmental events associated with the phenomenon of induction could be correlated in any way with special biochemical processes or properties in particular regions of the embryo. It is perhaps natural that some of the earliest efforts in this direction should concern the study of the overall metabolism of various regions of the gastrula as revealed by their total oxygen uptake. The first investigation was undertaken by Brachet (1935), when he measured the effects of localized cautery on the respiratory metabolism of *Rana temporaria* gastrulae. His results indicated that destruction of the cells in the vicinity of the dorsal lip produced a somewhat greater reduction of oxygen

* It is a pleasure to record my thanks to Dorothy Burgess Everett for her invaluable assistance in securing the data represented in this section on "The Development of Enzyme Systems in the Embryo," and for her careful preparation of the graphs in this paper.

consumption than when a corresponding area from the opposite side of the embryo was removed. Measurements of the carbon dioxide production of dorsal lip and ventral ectoderm explants yielded essentially similar results (Brachet, 1935). In these studies, quantitative estimations of the amount of tissue used in the comparative tests were not made, so that the results, although highly suggestive, had to be interpreted cautiously. However, in later work (Brachet, 1936), Kjeldahl nitrogen determinations were made on the explants of *Discoglossus* gastrulae after their carbon dioxide production had been measured, and the results indicated in this case that the rate of carbon dioxide production by pieces of gastrula from the dorsal lip region was unquestionably greater than that of ventral ectoderm.

At the same time, the oxygen consumption of explants of *Triton alpestris* gastrulae was measured by Waddington, Needham, and Brachet (1936) in a modified Gerard-Hartline respirometer. In most cases, the dorsal lip was found to have a greater oxygen consumption than the ventral ectoderm, but when respiration was related to dry weight no difference between dorsal lip and ventral ectoderm was noted.

By an ingenious method, Brachet and Shapiro (1937) measured simultaneously the oxygen consumption of the two halves of the intact gastrula of *Rana sylvatica*. The egg was placed in the center of a capillary, so that the respiration of one hemisphere could be directly compared with that of the other. When the gastrula was oriented so that its dorsal-ventral axis was perpendicular to the long axis of the capillary, no difference in the respiratory rates of the two halves of the egg was observed. However, when the dorsal lip region and ventral ectoderm were directed toward opposite ends of the capillary, the respiration of the hemisphere containing the dorsal lip was some 40 per cent greater than that of the opposite side. This does not necessarily indicate that the dorsal lip has a more intense metabolism than the opposite side of the gastrula, for, as Fischer and Hartwig (1938) have pointed out, it is unlikely that the index drops in the two limbs of the capillary were being influenced by equal amounts of respiring tissue.

Following this, Brachet (1939) further investigated the respiration of explants of gastrulae, using large numbers of pieces in the Meyerhof-Schmitt respirometer. Because of the insensitivity of the instrument, respiration had to be followed for 20 to 30 hours. Brachet's results indicated that the respiration of the dorsal lip region of *Discoglossus* gastrulae was, on the average, some 30 per cent greater than that of the ventral ectoderm. Carbon dioxide production averaged 84 per cent greater in the dorsal lip region. In a second series of experiments, Brachet (1939), using the Brachet-Shapiro apparatus for measurements on the intact gastrula, reported that the average value for the respiration of the dorsal lip-containing half of the gastrula of *Rana temporaria* was not significantly different from that of the half containing the ventral ectoderm. However, carbon dioxide production, measured on the intact

gastrula by a modification of the same method, was again seen to be much greater in the half containing the dorsal lip.

Fischer and Hartwig (1938), using as many as 60 explants in Warburg respirometers in experiments lasting from 16 to 23 hours, obtained an average figure for the respiration of the dorsal lip of 2.34 cu.mm. per 10 mg. dry weight per hour. The corresponding value for the ventral ectoderm was 1.83. In experiments of shorter duration, *i.e.*, 6 to 9 hours, the difference was less, the averages being respectively 2.13 and 1.92.

Using the Cartesian diver technique, Boell and Needham (1938, 1939) and Boell, Koch, and Needham (1939) measured the oxygen consumption of explants of dorsal lip and ventral ectoderm of two species of amphibia. In these experiments, made on single explants, the respiratory measurements were confined to a period of 3 hours following isolation of the explants and their insertion into the divers. These investigations showed no significant difference in the average respiratory rates of dorsal lip and ventral ectoderm, and confirmation of this result was obtained with *Rana temporaria* by Needham, Rogers, and Shen (1939). In our work, some variation was noted in the relative rates of respiration of the two kinds of tissue from experiment to experiment. Out of a total of 23 tests in which the tissue fragments whose respiration was being compared were isolated from the same gastrula, 9 showed the rate of oxygen uptake of the dorsal lip to be greater than that of ventral ectoderm. In 16 experiments the opposite was true, and in 3 cases the respiratory rates of the two regions were identical. Brachet (1945, p. 389) has suggested that the variation in our results may have been due to the effects of tissue cytolysis at the air-fluid interface because of the small amount (2 cu.mm.) of Holtfreter's solution used in the divers. However, calculation shows that, even with the largest tissue samples, the volume of saline in which the tissue was supported was about 8 times that of the tissue, and in the majority of the experiments this figure was nearer 20. Examination of the tissues in the divers at the end of the experiments revealed that they remained well-healed and could be removed intact for Kjeldahl determinations. Furthermore, in most cases the respiratory rate was constant during the experimental period. "*Ce serait*," states Brachet, "*un indice d'un état physiologique demeuré normal*." As will appear below, it is highly probable that the variation in our results was due to the fact that the dorsal lip and ventral ectoderm differed in composition from experiment to experiment.

The experiments which have been mentioned in the discussion thus far are summarized in TABLE 1. They were performed on embryonic material from different species and under various experimental conditions. It is unlikely, however, that such factors are solely responsible for the lack of agreement in the results. Brachet (1939) has stated that, in experiments of short duration, the respiratory rates of dorsal lip and ventral ectoderm are essentially identical, but that in longer experiments the respiratory rate of the dorsal lip is greater. This is due, he believes,

TABLE 1

SUMMARY OF WORK ON THE RESPIRATORY RATES OF DORSAL LIP AND VENTRAL ECTODERM IN THE AMPHIBIAN GASTRULA

Investigator	Material	Rate DL	Rate VE	DL/VE
Waddington, Needham, & Brachet	<i>T. alpestris</i> ¹	0.23	0.21	1.10
Brachet & Shapiro	<i>R. sylvatica</i> ²	85	58	1.47
Fischer & Hartwig	<i>A. mexicanum</i> ³	2.31	1.83	1.28
Fischer & Hartwig	<i>A. mexicanum</i> ³	2.13	1.92	1.11
Boell & Needham	<i>Discoglossus</i> ⁴	4.80	4.93	0.98
Boell & Needham	<i>A. mexicanum</i> ⁴	3.21	3.18	1.01
Boell, Koch, & Needham	<i>A. mexicanum</i> ⁴	5.3	4.2	1.26
Brachet	<i>R. temporaria</i> ⁵	0.164	0.153	1.07
Brachet	<i>Discoglossus</i> ⁴	4.1	3.1	1.31
Needham, Rogers, & Shen	<i>R. temporaria</i> ⁴	3.74	3.78	0.99

Respiratory rates (μ l. O₂ consumed per hour) are based on the following units of tissue: 1. milligram dry weight; 2. gram wet weight; 3. 10 milligrams dry weight; 4. milligram nitrogen; 5. not stated by the author.

to the fact that the dorsal lip tissue differentiates more completely after excision from the embryo than ventral ectoderm and that its respiratory rate therefore increases during the process, whereas that of ventral ectoderm does not. Although this seems a plausible explanation, it is likely that the variations in the results reported above are due even more to fundamental differences in technique. Perhaps the factor of greatest importance has been the failure of various workers to use corresponding areas of dorsal lip and ventral ectoderm for comparison. This is understandable, however, when it is remembered that the various presumptive areas of the gastrula have only statistical boundaries. So-called dorsal lip material varies in composition with the age of the gastrula. In the very youngest stages, it consists exclusively of chorda mesoderm, most of which has not yet been invaginated. In later stages, it is composed of a double layer of tissue, the outer one representing presumptive neural plate and the inner layer comprising chorda mesoderm or archenteron roof. In a given isolate of dorsal lip, the relative amounts of mesoderm and ectoderm will vary, accordingly, with the age of the gastrula from which it is taken as well as with the size of the explant. Furthermore, the term "ventral ectoderm" denotes no well-defined zone in the gastrula, and the respiration of a fragment of ectoderm may therefore be expected to vary somewhat, depending upon its position in the gastrula.

Regional Variation in Respiration of Gastrular Explants. In more recent work, some of these considerations have been taken into account, and comparisons have been made of the respiratory rates of presumptive regions taken from various levels of the gastrula (Boell and Nicholas, 1940; Boell, 1942; Barth, 1939, 1942). The results of these experiments are summarized in TABLE 2 and indicate that differences of considerable magnitude appear in the oxygen consumption of different areas from the same gastrula. There seems to be a gradient of respiratory activity

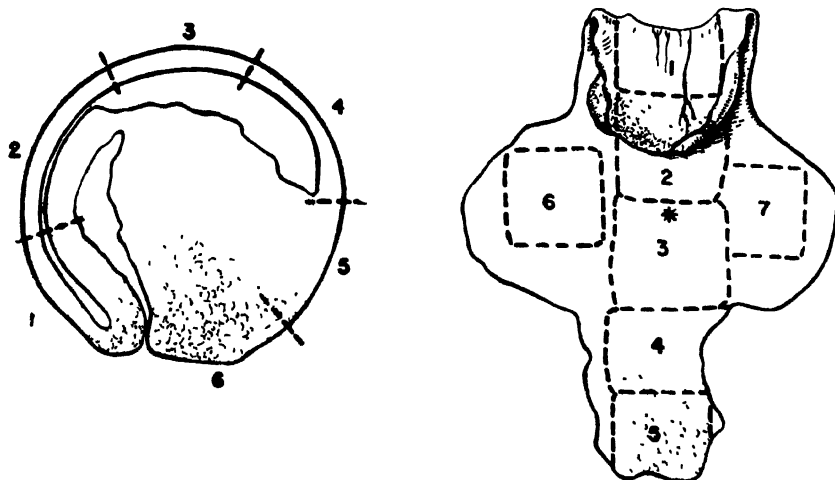


FIGURE 1. Diagram showing method of dissecting pieces of tissue used in the experiments summarized in FIGURE 2 and TABLE 2. Piece 1 refers to the dorsal lip region.

extending from the animal pole to the vegetal pole with the more anterior part of the presumptive neural plate showing the highest rate of respiration. As might be expected, some variation in the absolute levels of the various regions is apparent from gastrula to gastrula, but the general pattern of respiratory rate is similar to that indicated in FIGURE 2.

TABLE 2
RESPIRATION OF VARIOUS REGIONS OF THE GASTRULA

Region	No. expts.	$Q'O_2$	P E are.
Dorsal lip	31	2.1	0.07
Presumptive neural plate	14	4.9	0.98
Anterior ectoderm	20	4.5	0.16
Posterior ventral ectoderm	10	3.0	0.19
Yolk endoderm	13	1.3	0.09
Right lateral ectoderm	3	3.1	0.14
Left lateral ectoderm	6	3.4	0.31
Chorda mesoderm	6	1.2	0.09

Furthermore, it appears that the differences in respiration among the various regions are more marked when the pieces used for comparison are small and represent well-localized areas. The use of small pieces of tissue in respiration studies has been criticized by Child (1946, p. 127). It may be stated, however, that the pieces of tissue used in the work summarized in TABLE 2 were somewhat larger than those used routinely in transplantation experiments.

Barth (1942) has measured the oxygen uptake of explants of three species of Amphibia by means of a micro-Winkler technique. Although his measurements were made on several gastrular pieces of somewhat larger size than those in our experiments, the results obtained were

similar to those already mentioned. It thus appears that the relative respiratory rate of dorsal lip tissue depends upon the particular region

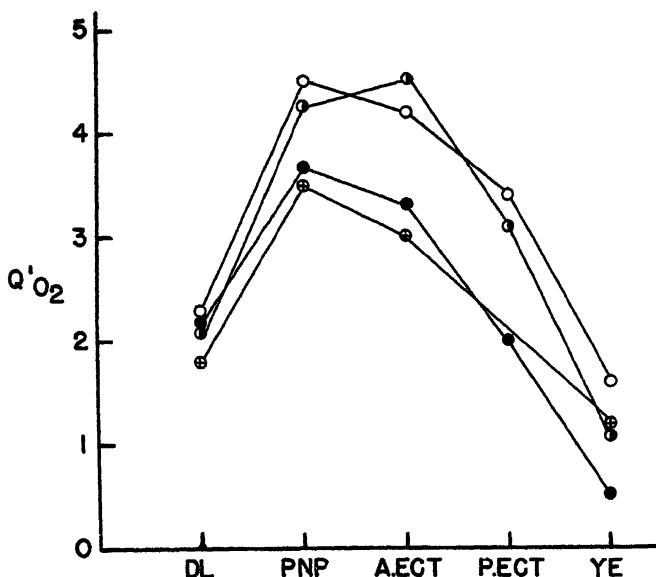


FIGURE 2. Graph showing the variation in the respiratory rates, $Q'O_2$, of various regions of the gastrula of *Amblystoma punctatum*. DL=dorsal lip material. PNP=presumptive neural plate. A.ECT=ectoderm from the region of the animal pole. P.ECT=ectoderm from region between animal and vegetal pole on ventral side of the gastrula. YE=yolk endoderm.

of the gastrula with which it is compared, being higher than that of ectoderm if the latter tissue is removed from near the vegetal pole, and lower if it is taken from the animal pole of the embryo. This factor is doubtless mainly responsible for the differences in the results of various workers reported in TABLE 1. Moreover, it seems the most likely explanation of the variation in the relative respiratory rates of dorsal lip and ventral ectoderm from gastrula to gastrula reported by Boell and Needham (1939).

Dorsal lip material represents a double layer of tissue while the other regions selected for study are generally thinner. One might question, therefore, whether sufficient oxygen from the air in the gas space of the divers was diffusing into the dorsal lip tissue to maintain respiration at its potential maximum. Therefore, a number of experiments were performed in which the divers were filled with oxygen instead of air and data obtained on the relative respiratory rates of the various regions of the gastrula under these conditions. Comparative figures are presented in TABLE 3. These experiments show clearly that the difference between dorsal lip and ectoderm is due to intrinsic dissimilarities in the tissues rather than to differences in the availability of oxygen. The lower respira-

TABLE 3
COMPARISON OF THE RESPIRATORY RATES OF REGIONS
OF THE GASTRULA IN AIR AND IN PURE OXYGEN

Region	Air		Oxygen	
	\dot{Q}'_{O_2}	Per cent	\dot{Q}'_{O_2}	Per cent
Dorsal lip	2.1	43	1.6	50
P. neural plate	4.9	100	3.2	100
Ant. ectoderm	4.5	92	3.2	100
Post. ectoderm	3.0	57	1.8	56
Yolk endoderm	1.3	27	0.8	25

atory rates in oxygen as compared with the average values in air are simply a reflection of the variation in oxygen uptake in different gastrulae. Pure oxygen, apparently, has no deleterious effect on the tissue explants, and it may be recalled that Parnas and Krasinka (1921) showed that pure oxygen was without effect on the respiration of intact embryos.

Regional Distribution of Yolk and the Respiratory Rate of Yolk-Free "Active" Material in the Gastrula. The amphibian egg contains a large amount of yolk which is distributed asymmetrically between the animal and vegetal poles (Bragg, 1939; Daniel and Yarwood, 1939). Although important as raw material in developmental processes, yolk is generally believed to be relatively inert metabolically. Pickford (1943) found that the dipeptidase activity of yolk is negligible, and Boell and Shen (1944) have shown that yolk obtained by centrifuging homogenates of whole amphibian embryos possessed very little cholinesterase activity. In view of the differences in the yolk content of the various regions of the gastrula, it seemed likely that the differences in respiratory rate might simply reflect the presence of varying amounts of yolk.

Accordingly, determinations of the yolk content of pieces of the amphibian gastrula corresponding to those used in respiration experiments were made (Barth, 1942; Boell, 1942). In the latter work, pieces of gastrula were centrifuged in the Beams air-turbine centrifuge after their initial volume had been determined. Centrifugation at high speeds completely disrupted the tissues and stratified their components so that the amount of yolk could be estimated volumetrically (FIGURE 3). The yolk content of the various regions of the gastrula is summarized in TABLE 4. These figures differ rather widely from the indirect determinations of Barth (1942), but accord with the older measurements of McClendon (cited by Needham, 1931), who found that about 78 per cent of the *Rana pipiens* egg is represented by the yolk fraction.

From the data in TABLES 2 and 4, it appears that the respiratory rate of a piece of tissue is inversely proportional to its yolk content or directly proportional to the so-called "active" material (FIGURE 4). If a direct relationship actually exists between respiration and active substance, one might reasonably expect the extrapolated curve in FIGURE 4 to intersect

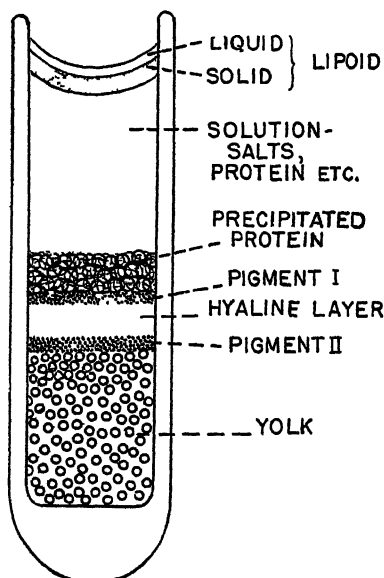


FIGURE 3. Diagrammatic representation of the stratification of gastrula explants after high-speed centrifugation.

the abscissa at zero. The fact that it does not do so presumably means that the "active" fraction still includes substances, such as lipoidal material and pigment, which are metabolically inert. It is interesting to note that, when respiration is corrected for the amount of inactive material indicated by the graph, the respiratory rates of different parts of the gastrula are approximately identical. It may be mentioned in passing that the inability to obtain, by direct means, a measure of the meta-

TABLE 4
YOLK CONTENT OF VARIOUS REGIONS OF THE GASTRULA
AND RESPIRATION OF "ACTIVE MATERIAL" OF THE TISSUE

<i>Region</i>	<i>No. deter- minations</i>	<i>Per cent yolk</i>	<i>Per cent active material</i>	<i>Resp.</i>
Dorsal lip	7	36	41	4.8
Presumptive neural plate	7	33	67	7.3
Anterior ectoderm	7	31	69	6.5
Posterior ectoderm	7	43	57	5.9
Yolk endoderm	8	66	34	3.8
Right lateral ectoderm	2	34	66	4.7
Left lateral ectoderm	2	34	66	5.1
Chorda	2	63	37	3.3

bologically active fraction of a piece of tissue emphasizes again the inadequacy of the usual units such as dry weight, total nitrogen, tissue volume, etc., as quantitative indices of the amount of embryonic material used in comparative tests of this kind.

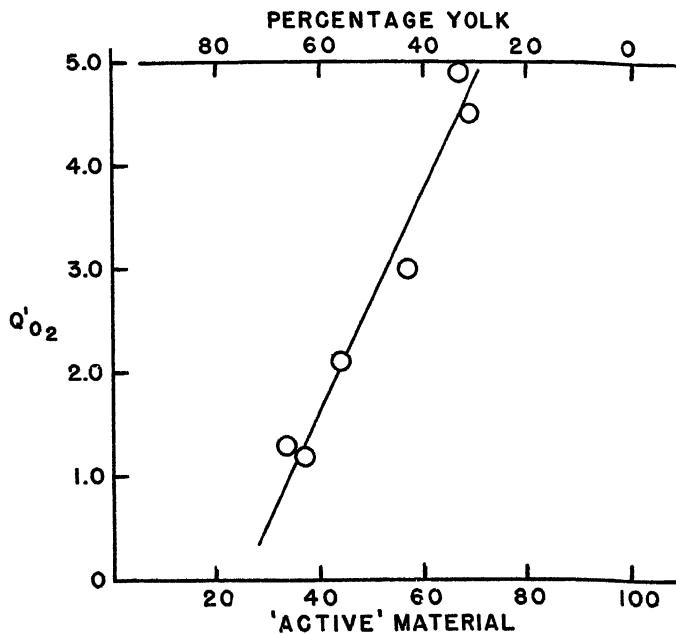


FIGURE 4. Graph showing a plot of the respiratory rate ($\text{m}\mu\text{l. O}_2/\mu\text{g. N}/\text{hour}$) of explants of various regions of the gastrula against the percentage yolk content of the regions.

The data summarized in TABLES 2 and 4 are of particular interest when considered in relation to Child's theory of axial gradients as it applies to organizer action. From the results of various non-quantitative tests (such as differential susceptibility to toxic substances, differential staining, etc.) which have been applied to the amphibian gastrula or to other developing or regenerating animals, Child (1929, pp. 50-51) has concluded that "... at the time of gastrulation the region about the dorsal lip is the region of most intense metabolic activity in the embryo and that its dominance as an organizer results from this condition." In addition, he has stated "... that various facts indicate a very great increase in metabolism during early stages of amphibian development up to the gastrula and that the dorsal lip region at the time of gastrulation is probably not only the region of most intense metabolism in the embryo at that stage, but also of most intense metabolism in embryonic development, because it is physiologically youngest at that stage." In other words, if these statements are interpreted correctly, Child contends that the organizer is an organizer "primarily because of its quantitatively greater activity" as compared with other parts of the embryo. More recently, the same author (1941) has again expressed this view in concluding that "in general, the natural inductors are, or at certain developmental stages, become, high gradient levels, and their inducing ac-

tion apparently consists in alteration of conditions in regions representing lower levels. The inductor apparently represents a higher range of gradient levels and by raising the level of presumptive neural plate, makes possible further development." Barth (1942) has shared the opinion that "the stimulus for organization proceeds from tissues exhibiting a high rate of oxygen consumption."

Needham (1942), on the other hand, has expressed the opinion that "although gradients of susceptibility to lethal agents, intensity of vital staining, etc., had been clearly demonstrated in a large number of adult invertebrates and many embryos, no evidence whatever has been brought forward to justify a belief in the existence of 'respiratory' or 'metabolic' gradients in embryos, and very little satisfactory evidence for this in the case of adult organisms." Furthermore, after reviewing the available data on the subject, Needham concludes that there is little basis for considering that respiratory gradients are in any way connected with the phenomenon of induction in the amphibian gastrula. Child (1946), in a further attempt to support his view, has stated, however, that the data on respiratory determinations of various gastrular regions "are, at present, far from agreement with Needham," and he rejects the determinations of Boell and Needham (1939), and presumably all others which fail to show a difference in the respiratory rates of dorsal lip tissue and ventral ectoderm, as being without definite significance since no account was taken of varying amounts of yolk in the pieces used in comparative tests. "The dorsal lip cells," he writes, "contain more yolk than those of ventral ectoderm. If they respire at the same rate without correction for yolk, the metabolizing protoplasm of the dorsal lip must actually respire at a considerably higher rate than that of ventral ectoderm." It is clear, however, from an examination of the data in TABLE 4, that even after correction for yolk content, the dorsal lip does not have a higher respiration than ventral ectoderm. A more significant comparison, from the standpoint of Child's theory, would be that between dorsal lip and presumptive neural plate. However, the data in TABLE 4, as well as those of Barth (1942), provide no support for the view that these regions of the gastrula represent respectively "higher" and "lower" gradient levels in the developing system. Furthermore, it would seem unlikely that a quantitative level of metabolism, or difference of level between inducing and induced tissues, is necessary, since it has been experimentally demonstrated that induction and neural differentiation can occur in the presence of cyanide in a concentration sufficient to inhibit respiration by 80 to 90 per cent (Brachet, 1939; Barnes, 1944; Boell, unpublished experiments) and under anaerobiosis (Brachet, 1939). It is of further interest to note that Phillips (1942) found no difference of significance in the respiratory rates of various presumptive regions of the early chick blastoderm. Moreover, Lindahl and Holter (1940) reported that the respiratory rates of animal and vegetal halves of sea urchin embryos (*Paracentrotus lividus*) were the same in spite of their different developmental

potentialities. Thus, there can be little doubt that inductive action is not associated with quantitatively higher respiratory metabolism in the dorsal lip than in the presumptive neural plate or in other parts of the gastrula.

Respiratory Quotients and Glycolysis. Studies by different workers on the respiratory quotients of various gastrula regions are in complete agreement in showing a value of approximately unity for the dorsal lip tissue and considerably lower values for ventral ectoderm. It has already been noted that Brachet observed, in comparative tests on dorsal lip material and ventral ectoderm, a greater difference in favor of the dorsal lip for carbon dioxide output than for oxygen uptake, and this was correctly interpreted as indicating a difference in the respiratory quotients of the two regions. Subsequent work, summarized in TABLE 5, has abundantly confirmed Brachet's initial observation.

TABLE 5
SUMMARY OF RESPIRATORY QUOTIENTS OF DORSAL LIP AND
VENTRAL ECTODERM FROM THE AMPHIBIAN GASTRULA

Investigator	Material	R.Q. DL	R.Q. VE
Brachet	<i>Discoglossus</i>	1.02	0.73
Brachet	<i>R. fusca</i>	0.97	0.80
Boell, Koch, & Needham	<i>A. mexicanum</i>	0.98	0.87
Needham, Rogers, & Shen	<i>R. temporaria</i>	0.92	0.81

Brachet (1934) has shown that the R.Q. of intact amphibian eggs averages approximately 0.66 during cleavage stages but rises abruptly to approximately 1.0 during gastrulation, at which point it then remains during subsequent development. Mendes (1948, in press) has extended the study of the respiratory quotient during development of the frog, *Rana pipiens*, to include all stages from immediately after fertilization to the end of the pre-feeding period. He has found that the R.Q. of newly fertilized eggs and of embryos in the early cleavage stages is unity. However, in the late blastula stage, the R.Q. drops to an average value of about 0.7, only to rise again during gastrulation.

Boell, Needham, and Rogers (1939) have shown that the rise observed by Brachet (1934) and Mendes occurs also in isolated regions of the embryo but values of unity are reached by the dorsal lip region in advance of the ventral ectoderm. In the case of the former tissue, the respiratory quotient becomes unity as soon as gastrulation commences, suggesting that the process of invagination is in some way associated with carbohydrate metabolism. Gastrulation in the ventral tissues, presumably, is also concerned with carbohydrate breakdown and an R.Q. of 1.0, but the process appears later in development than it does on the dorsal side of the embryo and it is probable that the respiratory quotient of ventral ectoderm does not reach unity until the tissue becomes underlain by mesoderm.

These respiratory quotients, although admittedly difficult to inter-

pret, have generally been regarded as indicating that carbohydrate metabolism predominates in the dorsal lip region and that ventral ectoderm shows a mixed metabolism. It should be recognized, however, that complete combustion of protein with ammonia as the nitrogenous end product will likewise yield a respiratory quotient near unity. However, it seems unlikely that this is the case in the dorsal lip region of the gastrula since it has been shown that very little ammonia is produced under aerobic circumstances by explants of dorsal lip (Brachet, 1939; Boell, Needham, and Rogers, 1939; Needham, Rogers, and Shen, 1939).

The conclusion which has been derived from the respiratory quotient data reported above, *viz.*, that gastrulation in some way involves carbohydrate breakdown, is made more tenable by direct determination of glycogen in various gastrular regions. Woerdemann (1933) first showed, by a histochemical method, that glycogen disappears from the dorsal lip tissue during invagination. Although the technique used in obtaining this result was not above criticism, Woerdemann's observation was confirmed by Heatley (1935) and Heatley and Lindahl (1937). These authors compared the glycogen content of corresponding regions in the blastula and gastrula of the axolotl embryo and found a significant decrease (31%) in glycogen only in the dorsal lip region (see TABLE 6).

TABLE 6
TOTAL GLYCOGEN IN THE AXOLOTL EMBRYO
Figures in mg. % dry weight
(From HEATLEY & LINDAHL, 1937)

Region	Blastula	Gastrula	Per cent decrease
D 1	17.8	16.5	7
D 2	12.0	8.3	31
D 3+V 3	4.3	3.9	9
V 1	16.7	16.5	1
V 2	10.0	9.3	7

Recently, Jaeger (1945) has confirmed this finding in analyses of *Rana pipiens* embryos (TABLE 7). It is interesting that Jaeger found approxi-

TABLE 7
TOTAL GLYCOGEN IN REGIONS OF FROG EMBRYO
Figures in mg. % dry weight
(From JAEGER, 1945)

Region	Before gastrulation	After gastrulation	Per cent decrease
DL <i>in situ</i>	12.3	8.2	33
VL <i>in situ</i>	14.0	10.1	28
DL explant	11.2	11.1	1
VL explant	10.8	10.3	4.5
DL+ECT explant	12.8	12.5	2.5

mately the same percentage decrease in glycogen (33%) in the dorsal lip

region as was observed by Heatley and Lindahl. Unfortunately, her analyses did not include a comparison with other parts of the gastrula during the same developmental period, but one may conclude, on the strength of Heatley and Lindahl's observations, that the decrease in other regions is perhaps small. Jaeger went on to show, in confirmation of Tanaka's (1934) earlier report, that glycogen was likewise lost from the ventral lip when it underwent invagination. Apparently, glycogen loss from these regions was not an "autonomous" process, for explants of dorsal and ventral lip in saline solutions showed no appreciable decrease in glycogen. Furthermore, glycogen was not lost from presumptive dorsal or ventral lip material in hybrid embryos in which developmental arrest occurred at the gastrula stage. Nor was glycogen loss associated with the induction of neural tissue, since no decrease of glycogen was observed in explants of dorsal lip and competent ectoderm in which neural differentiation had occurred (as indicated by histological appearance of the tissues). Glycogen loss seems not to be associated simply with the assumption of an "internal position" of the tissue after gastrulation, for Raven (1935 a and b) demonstrated that pieces of ectoderm implanted into the blastocoel of the amphibian embryo showed no decrease in their glycogen content. However, a piece of dorsal lip material implanted so as to go through its normal invaginative movements did lose glycogen. Furthermore, a piece of ectoderm implanted in the dorsal lip region and carried into the interior of the embryo by the invagination of the dorsal lip lost glycogen in the same way as did the dorsal lip. From these observations, as well as from those of Jaeger, it appears highly probable that glycogen disappearance is in some way connected with the changes in cell shape and with cell movements during gastrulation. It may be as Needham (1942, p. 191) has suggested, that more than coincidence is involved between the contractile appearance of the blastoporal cells and their utilization of glycogen.

It is noteworthy that Jaeger's figures for dorsal lip tissue were obtained by comparing corresponding regions in embryos at stages 10 and 12, and that those for ventral lip were obtained on stages 11 and 14. Jaeger gave no indication of the time required for development between these stages, but reference to Shumway's (1940) time table for normal development in *Rana pipiens* indicates that the time interval between stages 10 and 12 is 16 hours, whereas 28 hours are required for the embryo to advance from stage 11 to stage 14. Since during these times the quantitative decrease in glycogen was about the same in both regions, the rate of glycogen loss per unit of time is correspondingly greater, indeed almost twice as great, in the dorsal lip than in the ventral lip tissue. This result is interesting in comparison with the data of Boell, Needham, and Rogers (1939) showing that anaerobic glycolysis occurred both in dorsal lip and in ventral tissues of the embryo, but that the rate of glycolysis was about three times greater in the dorsal lip than in ventral ectoderm (TABLE 8).

TABLE 8

ANAEROBIC GLYCOLYSIS ($Q_L^{N_2}$) IN DORSAL LIP AND VENTRAL ECTODERM
OF THE AMPHIBIAN GASTRULA

	DL	VE
<i>Rana temporaria</i>	+0.63	+0.21
<i>Triton alpestris</i>	+0.42	+0.14

The Development of Enzyme Systems in the Embryo

The organic matter of the amphibian egg may be divided into two fractions. One of these is the protoplasmic or metabolically active portion; the other consists of storage material or yolk, and, as was indicated in the foregoing discussion, there is evidence that this fraction is relatively inert metabolically and serves mainly as a source of raw material for development. It is worthy of note that, during the period of development covering the first 600 hours after fertilization, *i.e.*, during the period of egg and larval development included in Harrison's normal stages, the embryo takes in no food. The total organic matter of the egg thus remains essentially constant; actually it decreases slightly as can be seen in data for dry weight (Dempster, 1930, 1933) or total nitrogen (Wills, 1936). However, during development, the relative proportion of protoplasmic material and yolk is constantly changing as the yolk becomes gradually transformed into new protoplasm and the products of protoplasmic activity. It would seem that the raw materials of the egg represented in the yolk are present in highly concentrated form and that their transformation into protoplasm involves considerable hydration (Gray, 1926; Dempster, 1930, 1933; Boell, 1945). Indeed the embryo of *Amblystoma punctatum* takes in, during its pre-feeding development, approximately 20 mg. of water, and the dry weight is reduced accordingly from 35 per cent at stage 18 to less than 10 per cent at stage 45.

Respiration of the Developing Amphibian Embryo. Unfortunately, we know nothing about the rate at which yolk is being transformed into protoplasm in the normal development of the amphibian embryo, since so far it has been impossible to determine the quantities of the two entities separately. However, an indirect indication of the process may be seen in the changing metabolic activity during development of the embryo as indicated by the respiratory rate (*cf.* Gray, 1927, 1929). A graphic summary of data relating oxygen consumption of *Amblystoma punctatum* to the developmental age of the embryos is seen in FIGURE 5. It is apparent, from the graph, that the amount of oxygen consumed by the embryo increases smoothly and steadily throughout development. Abrupt changes in the rate of respiration, or cyclic variations such as appear in the development of the sea urchin (Lindahl, 1939) or the grasshopper (Boell, 1935) are conspicuously absent. There is no indication of increased energy expenditure for such morphogenetic processes as gastrulation,

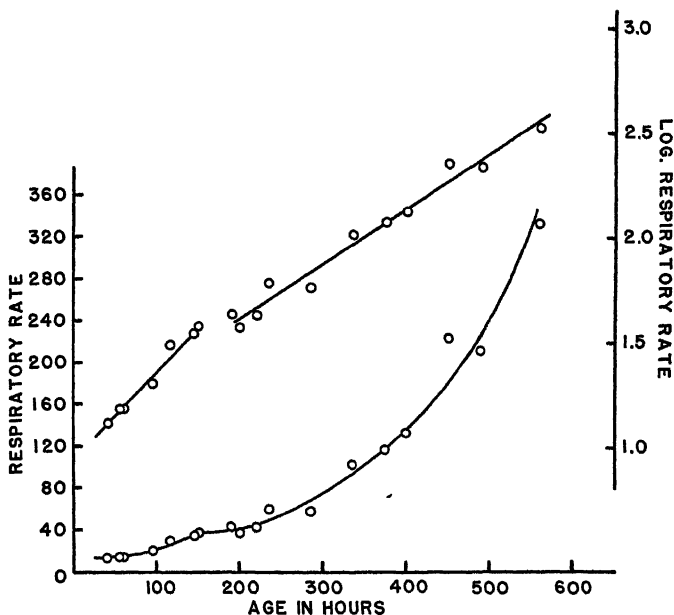


FIGURE 5. Graph showing the respiratory rate of intact embryos of *Amblystoma punctatum* during development. The arithmetic scale on the left relates to the lower curve; the logarithmic scale on the right, to the upper curve. Respiration is expressed as ml. O_2 /100 μ g. dry weight/hour.

the differentiation of the primary germ layers or their further elaboration into the definitive tissues and organs of the embryo. Obviously, any energy expenditure for these processes—since they themselves occur gradually—is merely represented as part of the general rise in oxygen consumption which occurs during development. Similar results have been obtained for a number of other amphibian species by Wills (1936), Atlas (1938), Fischer and Hartwig (1938), Hopkins and Handford (1943), Moog (1944), Barnes (1944), Spiegelman and Steinbach (1945), and Barth (1947). It may be concluded, then, that increase in respiration is a reflection of the progressive growth in the embryo of metabolically active material.

Growth is an exponential process (*cf.* Brody, 1945, chapter 16) and obeys the general relation $X = a \cdot e^{kt}$, or $\log X = \log a + kt (\log e)$. From this, it follows that any data which obey the equation should fall on a straight line when the logarithm of the growing entity is plotted against time. If respiratory increase during development is due to the growth of protoplasm, it would be expected that the course of respiration should increase exponentially. Curve B of FIGURE 5 indicates that this expectation is met. It is apparent in the curve that the rate of respiratory increase is not constant during development but occurs in two cycles with a “break” appearing on about the sixth or seventh day of develop-

ment, *i.e.*, at approximately stage 32 or 34. The values of k for the two cycles of growth are, respectively, 0.0098 and 0.0060 (see TABLE 9).

TABLE 9

TABLE OF CONSTANTS FOR THE GROWTH OF VARIOUS ENZYMES AND BIOCHEMICAL ENLITERS IN THE EMBRYOS OF *AMBLYSTOMA PUNCTATUM*

	k
Respiration—40 to 180 hours	0.0098
Respiration—180 to 360 hours	0.0060
Cytochrome oxidase*—60 to 360 hours	0.0075
Cytochrome oxidase†—115 to 630 hours	0.0075
Succinic oxidase—115 to 630 hours	0.0061
Cholinesterase—200 to 400 hours	0.0196
Cholinesterase—400 to 600 hours	0.0092
Volume of central nervous system—200 to 360 hours	0.0006

* *p*-Phenylenediamine used as substrate. Determinations in Warburg apparatus.

† Ascorbic acid used as substrate. Determinations in Cartesian diver apparatus.

Atlas (1938), Moog (1944), Barnes (1944), and Spiegelman and Steinbach (1945) have reported a similar situation in the respiration of *Rana pipiens* and *Rana sylvatica*, but in these species the break occurs after the end of gastrulation, that is, much earlier than in the salamander embryo. A semilogarithmic plot of Barth's (1947) data for *Rana pipiens* similarly shows a break at about stage 15 (Shumway's table). The older data of Bialasiewicz and Bledovski (1915, data taken from Needham, 1931, p. 677) likewise can be expressed by the equation mentioned above. In this work, the inflection in rate occurs at about the time when the external gills make their appearance, *i.e.*, at about 100 hours of development.

A semilogarithmic plot of the data for *Amblystoma tigrinum* (Hopkins and Handford, 1943) and *Amblystoma mexicanum* (Fischer and Hartwig, 1938) shows a break at about the same stage as in *Amblystoma punctatum*. Thus, it appears that a difference in the appearance of the break is characteristic of Urodeles and Anurans. It would be of interest to investigate this point further. It may be mentioned, in passing, that a similar inflection in the respiratory rate of the chick embryo can be seen when the data of Murray and Hasselbalch (Needham, 1931, Figure 156, p. 717) and Romanoff (1941) are plotted on a semilogarithmic scale. In the chick, the change in rate occurs at about the seventh day of incubation.

The fundamental cause of the break in the respiratory curves for these forms remains obscure, although it can hardly be doubted that the changing slopes of the curves reflect in some way the rate of transformation of yolk into active protoplasm. It is of considerable interest that in *Amblystoma* the change in rate of respiratory increase coincides with the time when the embryo begins to deviate markedly from the spherical form, and occurs only slightly before the initiation of the heartbeat and the establishment of circulation. Perhaps the rate of respiratory increase

is limited to some extent by the development of respiratory surface as well as by the maturation of the circulatory system as an oxygen carrier and as a means of transporting raw materials from the yolk mass to the rest of the embryo.

Cytochrome Oxidase. It has been shown by a number of investigators that the amphibian egg is highly sensitive to cyanide (Brachet, 1934; Barnes, 1944; Boell, 1945), and it has been generally concluded that respiration in the embryos of Amphibia proceeds by way of the cytochrome-cytochrome oxidase system. Measurements of the cytochrome oxidase activity of *Amblystoma punctatum* embryos (Boell, 1945) show that there is sufficient activity of this enzyme during all stages of development to account for the respiration of the embryos. FIGURE 6, curve A, illustrates that the increase in cytochrome oxidase activity during development follows a course similar to that of respiration, and like respiration increases exponentially with time. The similarity in the shapes of these curves, as well as the marked sensitivity to cyanide, thus suggest strongly that respiration is largely mediated by the cytochrome-cytochrome oxidase system during development. However, the concentration of cytochrome oxidase apparently is not the factor which limits the rate of increase in respiration, since k for growth of cytochrome oxidase is 0.0075 while for respiration k is 0.0060.

Spiegelman and Steinbach (1945) have reached a similar conclusion from a study of cytochrome oxidase in *Rana pipiens* embryos. These authors report that, although increase in respiratory rate occurs, cytochrome oxidase activity remains uniformly high throughout development and that enzyme synthesis apparently does not take place. This result is somewhat surprising, for it suggests that the unfertilized egg contains the full complement of cytochrome oxidase necessary for the embryo throughout development and that during the process the enzyme merely becomes distributed to the various cells and tissues of the growing organism. Furthermore, synthesis of the enzyme has been demonstrated in the embryos of other forms, as, for example, the grasshopper (Bodine and Boell, 1935; Allen, 1940) and the chick (Albaum and Worley, 1942; Albaum, Novikoff, and Ogur, 1946). Moreover, it may be mentioned that Spiegelman and Steinbach made measurements on embryos only up to stage 19 of Shumway's (1940) table which corresponds approximately with Harrison's stage 34 or 35 for *Amblystoma punctatum*. In *Amblystoma*, the major increase in enzyme activity occurs after this stage, and unpublished experiments of the author indicate that an increase in cytochrome oxidase occurs during the comparable period of development in *Rana pipiens* as well. These experiments show, in addition, that determinations of cytochrome oxidase in early stages of *Rana pipiens* are complicated by the fact that reducing substances in the egg homogenates apparently interfere with the oxidation of *p*-phenylenediamine (compare Brachet, 1945, p. 350), and the full activity of

the enzyme may not be obtained by the usual methods. It is interesting to note that Brachet's (1934) paper shows that *p*-phenylenediamine actually decreased slightly the oxygen consumption of homogenates of *Rana temporaria* eggs.

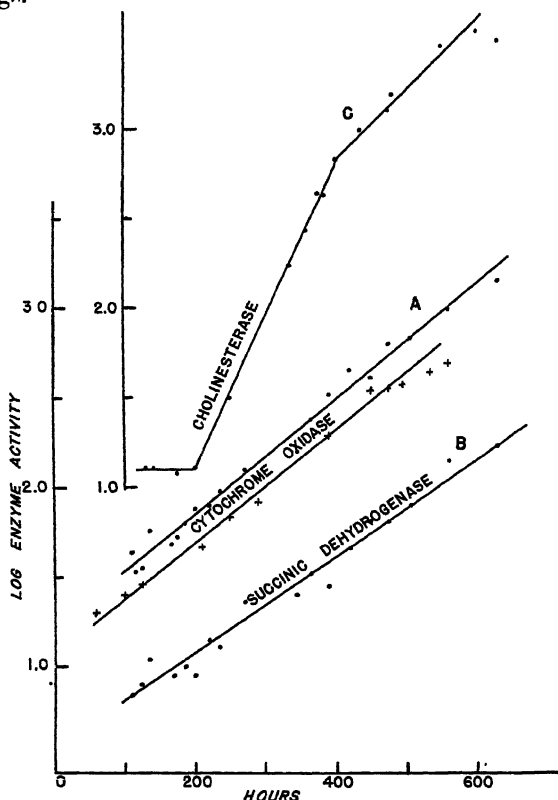


FIGURE 6. Semilogarithmic plot of enzyme activity against age of embryos of *Amblystoma punctatum*. The right-hand ordinate relates to cholinesterase activity, the curve for which has been shifted vertically to avoid superposition of curves. Enzyme activity is expressed as μ mol. gas produced or consumed in the Cartesian diver apparatus/100 μ g. dry weight/hour.

Succinic Oxidase. FIGURE 6, curve B, likewise shows that synthesis of succinic oxidase, as measured by the method of Schneider and Potter (1943), occurs throughout development (Boell, 1946a). Between 100 hours after fertilization and the end of the pre-feeding period (stage 46), succinic oxidase activity increases exponentially with time, the growth constant, k , being 0.0061. This is significantly less than the constant for cytochrome oxidase, but practically identical with that of the respiration of intact embryos during the same period of development. It is of interest that a break, comparable to that for respiration, is not apparent in the growth curves for either of these enzymes.

Schneider and Potter (1943) assume that measurement of the succinic oxidase system by their method actually determines the activity of succinic dehydrogenase. It is fairly certain that the limiting factor in the overall rate of oxygen uptake is neither cytochrome oxidase nor cytochrome, since the former is usually present in excess in the embryo homogenates and the latter can be supplied in excess by the addition of cytochrome *c*, but the method is incapable of distinguishing whether succinic dehydrogenase or some intermediary between succinic dehydrogenase and cytochrome *c* is the limiting factor in the reaction. It will be interesting to have data on succinic dehydrogenase activity alone by the ferrieyanide method of Quastel and Wheatley (1938), in order to gain information on this point. When such data are at hand, it may be possible to assess the significance of the identity in growth rates of respiratory and succinic oxidase activities.

Cholinesterase. So far, in this discussion, evidence has been presented in support of the view that increase in respiratory and enzyme activity during development is a reflection, and to some extent a measure, of the production of metabolically active protein from the storage proteins and other materials in yolk. The question naturally arises as to whether the growth of all enzymes may be expected to follow a similar course or whether there is evidence of specialization and individuality in these processes of biochemical differentiation. The results of a study of the development of cholinesterase activity in the embryo of *Amblystoma punctatum* provides interesting information along this line. FIGURE 7, taken from Sawyer's (1943) paper, shows that cholinesterase activity increases progressively throughout the major part of the developmental process. In premotile stages, the increase in cholinesterase activity is slight, but at the time of beginning motility in the embryos it increases abruptly. From a correlation of the development of behavior according to the pattern described by Coghill (1929) and the development of cholinesterase activity, Sawyer was led to conclude that functional maturation of the neuromuscular apparatus of *Amblystoma* coincides with the development of cholinesterase to a quantitatively high level.

The shape of the curve in FIGURE 7 is similar, in some respects, to the curves relating respiration, cytochrome oxidase, and succinic oxidase to time of development. However, closer examination of these curves reveals that they are only superficially similar. We have repeated Sawyer's work in assaying the cholinesterase activity of embryos during development and have fully confirmed his observations. As curve *C* of FIGURE 6 shows, there is essentially no increase in cholinesterase activity during the period of premotile development between 130 and 200 hours (from stage 25 to stage 34). However, at the time when the embryo is first capable of making rapid, repetitive movements, cholinesterase activity is considerable and the enzyme increases in activity throughout the remainder of the period of development studied. The rate of cho-

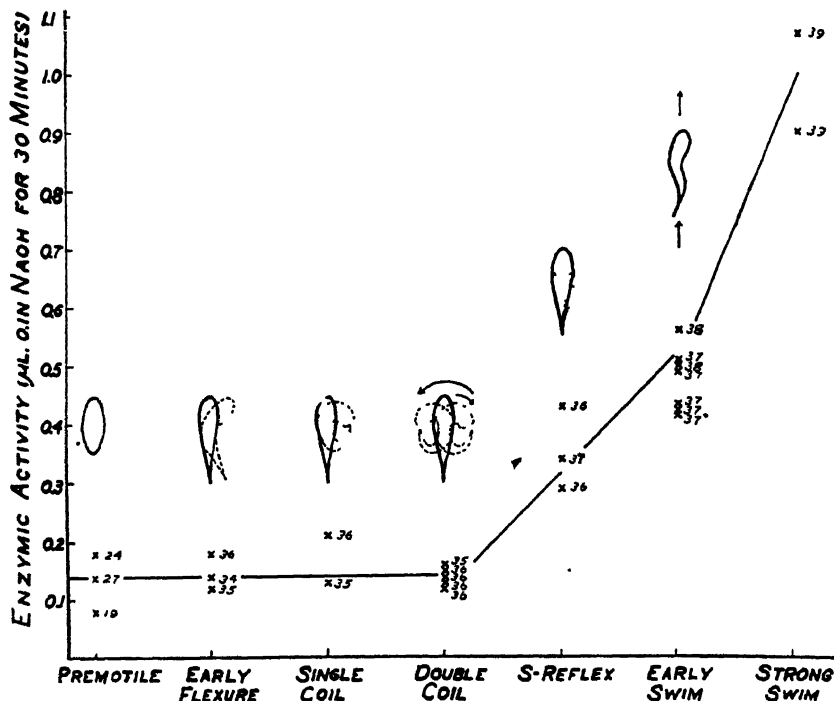


FIGURE 7. Summary of Sawyer's (1943) study of the relationship between the development of behavior and cholinesterase in *Amblystoma punctatum*. The numbers opposite the experimental points denote Harrison's stages.

linesterase synthesis is not constant, however, throughout the entire period. A break in the growth curve occurs after 400 hours or at approximately stage 41-42. Before the inflection, k is 0.0196, afterwards it is 0.0092. It is of considerable interest to note that the time at which the break occurs is correlated with decreased responsiveness of the embryo to mechanical stimulation, as shown in Detwiler's (1946 a and b) studies.

It would appear, from the differences in their growth rates, that the various processes of biochemical differentiation described in the foregoing discussion have certain specific and individual characteristics. If it be true that the increase in activity of respiration and respiratory enzymes reflects an increase in metabolically active material, it seems equally true that the changes in cholinesterase activity do not. The measurements of cholinesterase activity mentioned above were made on homogenates of whole embryos, but there is evidence that the enzyme is associated, at least in early stages, with the development of the nervous system. Sawyer (1943) and Boell and Shen (1944), in comparative studies of the enzyme activity of neural and non-neural tissues, were

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able to demonstrate a marked localization of the enzyme in the nervous system as early as stage 19 (closing neural folds), and that the difference between neural and non-neural tissues became progressively greater during subsequent development. Furthermore, assays of cholinesterase activity in secondary nervous systems, produced experimentally by the inductive action of dorsal lip tissue (chorda mesoderm) which was implanted into the blastocoel of host embryos, gave unmistakable evidence of an increased concentration of cholinesterase in the differentiating nervous system (TABLE 10; Boell and Shen, 1944). In this study, the re-

TABLE 10
CHOLINESTERASE IN EXTRACTS OF PRIMARY AND SECONDARY NEURAL TISSUES
(From BOELL & SHEN, 1944)

Stage	Q' ACH		
	Primary nervous system	Secondary nervous system	Host ectoderm
26	+ 32	+ 38	+ 22
34	+ 15	+ 17	+ 6
34	+ 66	+ 58	+ 14

Q'ACH = μ mol. CO₂ produced in hydrolysis of acetylcholine by extract containing 1 μ g. N per hour.

sults indicated that the cholinesterase values for the secondarily induced nervous system were quantitatively of the same order as those of the primary nervous system of the embryo and considerably higher than those of ectoderm or skin. Thus, these experiments indicate that the phenomenon of induction alters the biochemical as well as the morphological fate of the induced tissue.

During development, the nervous system increases in size, and one is therefore led to inquire whether the increase in cholinesterase activity in the embryo may be simply due to the growth of the nervous system in volume. It is obviously impossible to obtain a measure of all the neural tissues in the embryo, because of the development of nerves and other peripheral elements, but it was thought that some information on this point could be gained by making a study of the growth in volume of the central nervous system. The data were secured by projecting 10 μ serial sections of *Amblystoma* embryos at a magnification of 150 times. The outlines of the central nervous system were drawn on paper and the areas of the tracings determined by means of a planimeter. In the head and trunk region, each section was drawn and analyzed, but in the tail, where the diameter of the spinal cord was fairly uniform over relatively long distances, only every fourth or eighth section was measured. The sum of the planimetric readings was then taken as a measure of the volume of the central nervous system at each stage. Although the shrinking of nervous tissue in fixative may not be uniform at all stages of development, it was felt, nevertheless, that this method of determining the volume of the nervous system in the embryo yields data which are infinitely more accurate than any which could be obtained from dis-

sected and weighed organs. FIGURE 8 indicates that the growth of the nervous system is essentially uniform between stages 34 and 46 and occurs at a relatively low rate; indeed, the rate of growth is only one-thirtieth

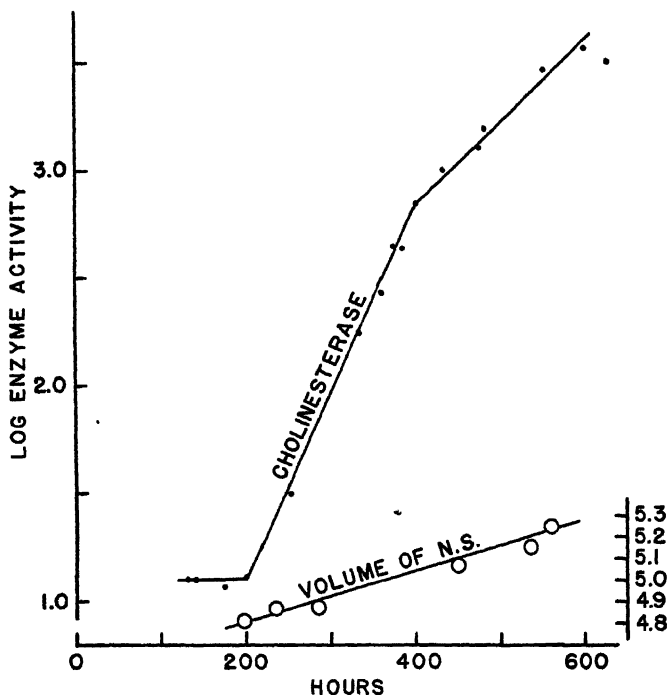


FIGURE 8. Curves showing the relative growth rates of cholinesterase activity and the volume of the central nervous system in *Amblystoma punctatum*. The upper curve is the same as curve C in FIGURE 6.

that of cholinesterase. In addition, inspection of the curves shows that they are qualitatively quite different. It thus appears that the increase in the concentration of cholinesterase is not simply a function of the growth in volume of the central nervous system. No doubt, the increase in enzyme activity parallels, to some extent, morphological and physiological differentiation of the nervous system. However, part of the increase of enzyme content of the whole embryo is unquestionably due to the development of cholinesterase in non-neural tissues of the embryo, for example, in muscle (Sawyer, 1943).

The Effect of Di-isopropyl Fluorophosphate. Di-isopropyl fluorophosphate (DFP) is one of a group of alkyl fluorophosphates studied by the chemical warfare services of both this country and England during the last war. According to Dixon and Needham (1946), DFP was found to inhibit cholinesterase and other esterases but had no effect on the activity of a number of other enzymes. The compound is characterized by its

intense anticholinesterasic activity, a concentration as low as 10^{-11} M producing a definite effect. Dixon and Needham conclude that the compounds to which DFP belongs "are the most powerful and specific enzyme inhibitors known." Mazur and Bodansky (1945) reported that the inhibition produced by DFP was irreversible as judged by the inability of serum esterase to regain its activity after prolonged dialysis (however, see Bulloch, Grundfest, Nachmansohn, Rothenberg, and Sterling, 1946). They also showed that regeneration of cholinesterase activity in the nervous systems of experimental animals treated with DFP required an extremely long time. In rabbit brain, for example, cholinesterase activity was only 90 per cent of normal 50 days after treatment.

In view of these properties of the compound, it was felt that treatment of *Amblystoma* embryos with DFP so as to produce what has been aptly termed a "biochemical lesion" might yield interesting information on the functional importance of cholinesterase (Boell, 1946b). It may be recalled that Sawyer (1943) had shown that the behavior responses of *Amblystoma* embryos were depressed in the presence of the reversible inhibitor, physostigmine, and that there was a rough correlation between depression of the enzyme and the loss of normal behavioral ability.

Embryos placed in DFP in premotile stages could apparently be reared indefinitely in a concentration of 0.0001 M with no effect on morphogenesis other than slight retardation of development. However, stronger concentrations were invariably toxic and resulted in the death of the embryos in a few minutes to a few hours. In a few embryos in 0.0001 M DFP, certain abnormalities of development were noted, but in the main the DFP-treated embryos seemed to be completely normal on the basis of external appearance. Moreover, the embryos, even when reared continuously in DFP solutions for a week or more, manifested the usual behavior reactions, and, at the appropriate stages (37-38), responded to tactile stimulation with swimming movements. However, the DFP-treated embryos, although apparently normal in appearance, fatigued more readily than control animals when stimulated, and when tested by Detwiler's (1946a and b) method for measuring behavior responses quantitatively they gave evidence of greatly reduced activity. After such embryos had been subjected to stimulation, they were usually incapable of responding again until a rest period of considerable length had elapsed. It is interesting to compare these results with the observations of Modell and Krop (1946). These investigators showed that cats that had been treated with DFP were perfectly normal in appearance and were well groomed but on stimulation gave evidence of marked myoneural disfunction. After a few steps, the animals fell over and could not be made to move again even when given a stimulus strong enough to elicit a cry.

Embryos reared in DFP solutions and capable of responding to stimulation were found to possess cholinesterase activity of considerable magnitude, although it was much lower than in control embryos of the same developmental stage. It is of some significance to note that the concen-

tration of cholinesterase in embryos reared in DFP was never less and usually greater than in normal animals at the time when they were first capable of responding to stimulation with rapid, repetitive movements. This is shown in TABLE 11 and would seem to indicate that a considerable

TABLE 11
CHOLINESTERASE ACTIVITY OF *Amblystoma* EMBRYOS
REARED IN 0.0001 M DFP

Days in DFP	Stage of embryo		Cholinesterase activity		Response to stimulation
	When DFP was added	When tested	Control	Treated	
1	25	26	13	0	No motility
2	25	26+	13	0	No motility
—	—	37	32	—	Rapid flutter
—	—	38	63	—	Swimming response
10	22	40	436	65	Embryo swims; tires
7	27	40	436	87	Embryo swims; tires
12	20	41	973	34	Rapid flutter
14	20	42	1100	48	Behavior not noted
11	27	42+	1540	163	Embryo swims; tires
17	22	45—	2860	855	Embryo swims; tires

excess of cholinesterase over that needed for minimal activity is present in the embryos at most stages (*cf.* Bulloch *et al.*, 1946). Under conditions of stress, the reduced cholinesterase activity may, however, lead to functional abnormality. This is clearly shown in a study of the effects of stimulation on the heart rate of the embryos. When normal embryos are stimulated until they are incapable of responding, the rate of heartbeat usually increases, but continued stimulation of DFP-treated embryos leads to a marked reduction in the rate of heartbeat. However, the beat is again normal after several minutes. Perhaps the residual cholinesterase in the tissues of the treated embryos is insufficient to remove the acetylcholine produced under such conditions of extreme activity. As a consequence, it may diffuse into the blood stream and be carried to the heart where it produces its characteristic depressing effect.

Apparently, synthesis of cholinesterase can take place in the embryo in the presence of DFP. TABLE 11 shows that the cholinesterase activity of embryos treated with DFP during premitile stages and reared continuously in DFP solutions increases five-to six-fold during the course of a week to ten days. After 17 days in DFP solutions, the cholinesterase activity may be as much as 65 times greater than in premitile embryos. Although the absolute level of enzyme activity is much lower in treated than in untreated animals of the same morphological stage, being on the average only 10 per cent of the control values, the rate of increase with development is approximately the same in the two groups of embryos. Apparently, therefore, development of the enzyme is not interfered with by DFP, although the newly synthesized enzyme is inhibited as soon as it is formed. The fact that the percentage enzyme activity of the

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treated animals in relation to the controls is approximately the same at all stages of development, suggests that an equilibrium exists between inhibited and uninhibited enzyme.

Summary

The foregoing discussion has dealt primarily with the demonstration of regional biochemical differences in the amphibian gastrula and with the increase in activity of certain enzyme systems throughout embryonic life. Processes of the latter type may be regarded as examples of biochemical differentiation since they represent special aspects of the synthetic or formative activities of the embryo. The discipline of chemical embryology is relatively young, and, although much progress has been made in the field in the past two or three decades, our knowledge of biochemical aspects of embryonic development is still relatively meager. It may be hoped that future work will supply us not only with more information on biochemical differences and differentiation in the embryo, but, in addition, with increased understanding of the way in which biochemical and morphogenetic processes are integrated in the complex series of events which characterize the transformation of the egg into the individual.

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FORM CHANGES DURING PRE-GASTRULAR DEVELOPMENT

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THE importance of movements of pre-gastrular materials has long been the subject of analysis by embryologists. The earliest observations on eggs have been those of studying cortical movements, movements of the materials which evidence themselves upon the surface of the egg. These movements and rearrangements are mirrored in the distribution of potencies and potentialities of the whole system in the later embryo. As is well known, Roux's (1885) early studies indicated a concept of fixity within the egg recently employed by Hamburger (1947) in an attempt to give a definite description of prelocalization at the time of fertilization or very shortly thereafter. It has been known since the time of Roux that, in certain forms, fertilization by means of an artificial insemination caused the formation of the grey crescent at a point opposite the region of sperm entrance; that the original cleavage bisects the grey crescent and coincides with the longitudinal axis of the embryo. This fixity has since been challenged and has been found not to exist in many of the other forms. It can be said with safety that there is no absolute fixed relation between the entrance of the sperm, the first cleavage, and the future axis of the embryo. While these coincidences may occur occasionally, according to statistical probabilities, they are not an interlocking determinant series.

The cortical movements of the egg have been most recently studied by Holtfreter (1944). He finds that, even in the unfertilized egg, one may have many of the cortical movements which are so characteristic of the fertilized egg. The movements of the pigment, the characteristic stranding of the pigment particles, the dispersion of pigment in certain areas—all these are obtained in unfertilized eggs. There may even be an attempt at a pseudo-gastrulation in which the surface parts of the egg, without having undergone any division at all, form lips similar to those which are found in gastrulation of a normal, fertilized type. It is true that these will not carry further, and since the material is still in a much more homogeneous condition, the materials of development are not specifically localized for further development of the organism. Holtfreter has repeated and amplified the explanations of Rhumbler (1902) in attempting to correlate the early rearrangements with the later surface movements which are so important in the allocation of materials.

Recently, there has been a renewal of the study in analyzing the movements and positions of parts in the interior of the egg. Daniel and Yarwood (1939) studied the relationship of parts in the various stages of the

egg beginning with the ovarian egg, then the coelomic egg, the oviducal egg, and finally the zygote. In each of these, there is a definite stratification of the yolk. The animal pole contains smaller granules than are found in each of the succeeding fifths, until one approaches the vegetal pole where there is an increasing aggregation of the smaller particles. This is in distinct violation of our generally expressed ideas that the vegetal pole of the egg contains all the large yolk-laden elements practically devoid of pigment, and is filled with yolk platelets from which they gain the distensibility of the membrane around the yolk packets. One fails to consider that the endodermal cells at the vegetal pole of the egg are vital, tangible, and mobile elements in the picture of later development. If one surveys the figure which is given by Daniel and Yarwood (1939), it is evident that the median three-fifths of the egg are the ones which contain the heavier amounts of the yolk particles, that the upper fifth and the lower fifth, that is, the animal pole and the vegetal pole, are much less heavily laden with the yolk material. It is particularly so in the zygote stage when fertilization has taken place and the materials are beginning to separate in a much more definitive fashion than they have in the ovarian, coelomic, or oviducal egg.

Schechtman (1934, 1935, 1937), in studying ingression, was the first to notice that material could pass from the outside of the egg in the region of the vegetal pole toward the inside of the egg and, if stained in the uncleaved egg, the stains placed upon the outside to mark the regions which ingress appeared in a columnar form extending to the floor of the blastocoele. This observation is of great importance, for it shows the movement of materials from the outside to the inside of the egg. It also shows their definite alignment along a certain, particular portion of the egg. It shows, in addition, that the movement is limited to rather early stages, since in the early blastula the stain, placed as a spot on the vegetal pole of the uncleaved egg, extends from the outside to the floor of the blastocoele, but if the stain is similarly placed in the late blastula, it appears only in the lower third of the blastular floor. This observation of Schechtman is so striking that it seemed necessary to repeat the staining experiments using different degrees of stain and following the material through to a later stage of development. The phenomena of ingression are proved definitely by Schechtman's work. Its import, however, was not clearly realized by him, and since the interpretation of the significance of the cells found in the ingressed mass must remain for future study, it is necessary to repeat and amplify Schechtman's work in order to find out exactly where the cellular elements bearing the stain are forming. For this reason, the stains were applied to the outside of the egg in a manner similar to Schechtman's, beginning with the unfertilized egg and running a series similar to his. The results are quite interesting in that they show that, if an intense stain is placed upon the uncleaved egg, one can perceive as development continues that there are two lines by which the stain is sent to the inside of the embryo. The first

is the one which Schechtman found and which characteristically occupies the central plane of the egg. It is as though one had a central axis of polarity in the amphibian egg due to the ingression of materials which were originally on the outside. It forms a distinct entity extending upward toward the blastocoele as a definite cone of stained material.

At the time of the late blastula, there are certain characteristic changes in organization of the interior which bring about a change in the axes of the embryo. At this time, one finds that the secondary part of the stain which had remained on the outside of the egg is carried around to the ventral lip of the blastopore, and then progresses with the ventral lip of the blastopore across the floor of the gastrocoele. The primary ingression cone has left its imprint on the center of the floor of the blastocoele and, as the gastrocoele encroaches upon the blastocoele, we find that the two colored areas approach each other as the blastocoele begins to lose its form and undergoes definitive progression toward amalgamation with the gastrocoele. In *Amblystoma punctatum*, the blastocoele remains and does not become continuous with the gastrocoele, but the stain which has come into the embryo from the ventral lip of the blastopore progresses forward in the floor of the gastrocoele until it unites with the stain which has come in through the primary ingressive mechanism.

If one applies stain to the embryo during the late blastula stage, the effect of the primary ingression with the cone of material extending inward toward the blastocoele floor is completely lost and one secures only the secondary migration of material which comes in through the ventral lip of the blastopore to the gastrocoele floor. It has been found (Nicholas, 1945) that the material which comes in through the primary ingression lies in the anterior region just under the floor of the foregut marking the region between the heart and the liver in the early stages. This is an important location and probably marks an area in which induction effects are taking place. In recapitulation, then, the staining experiments show definitely that Schechtman's primary ingression is a definite entity to which can be added, with an intensification of the stain, a secondary situation by which the stain will progress through the ventral lip of the blastopore and later join with those fragments of stain which are left from the primary ingression. Recently, Nieuwkoop (1946) has repeated Schechtman's experiments and shown the secondary relationship in an exceedingly clear way. Nieuwkoop interprets the secondary portion of the ingression which comes in through the ventral lip of the blastopore as lying in the surface of the midgut. The primary ingression coincides with the material which Schechtman found in *Triturus* and Nicholas in *Amblystoma*.

It is clear that polar orientation is influenced by the early cortical movements which, in themselves, are a reflection of the reorientation of the materials within the egg. Polar orientation has been said to occur in the ovary (Child, 1941), and undoubtedly in the amphibian egg this

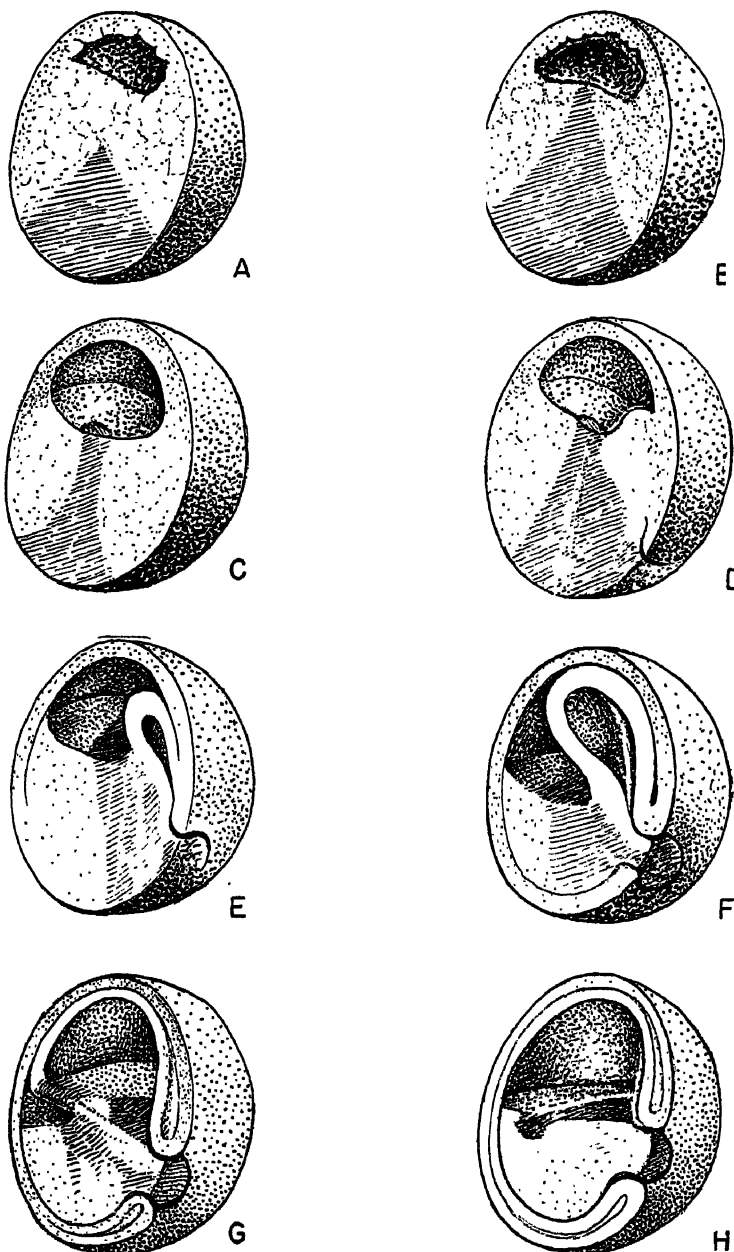


FIGURE 1. (For description see facing page)

is partially correct. There is, however, a marked deviation in the animal-vegetal axis shortly before fertilization and continuing afterwards. This may result in the marking of the axis 30 degrees from the original line at the time that the egg was laid. This reaction has been neglected to a large extent because we have not observed the difference in the polarity of the egg before fertilization and afterwards, but if one watches the egg one can see the change in the polarity of the animal-vegetal axis shortly after fertilization and to some extent before then.

The ingressive phenomena are essentially those which lead to the orientation of the mass within the egg after the early fertilization change in the polar axis. The inertness of the egg material which had formerly been thought to be burdened down by yolk has been found to be a fallacy in that an active movement of particles and materials does occur throughout the early stages before segmentation and apparently determines, to some extent at least, the relationship of the cortico-vegetal orientation.

The converse of this type of reaction is seen in the capacity of the embryo to undergo a tremendous amount of development without any of its yolk taking part in the reaction. This is true if one takes into account the fact that the yolk is undergoing a development of its own and has been in contact with the ectodermal and mesodermal portions of the system during their development. Nieuwkoop (1946) removed all of the yolk in the early neurula stage of amphibian embryos. When all of the yolk mass is removed from the neurula, it leaves the mesoderm which has moved inside through the blastopore, the entire chorda mesoderm which has underlain the nervous system, and the lateral mesoderm in its normal position. All of these migrations have been completed before the stage of the neurula. When the yolk is removed in this way, an embryo develops which is deficient in the head region, lacks gills, stomodaeum, all of the intestinal tract and its derivatives, and also the heart. Most of the nervous system, the lateral body wall and musculature are intact, but there is no splanchnopleure whatsoever. That is, the material which was destined to form smooth musculature of the gut is not formed. This speaks for a strong dependence of smooth musculature formation upon the gut structures which it normally surrounds. Another interesting phenomenon occurring after this operation is that two full sets

FIGURE 1. A (stage 5). The stain placed upon the vegetal region of the uncleaved egg extends upward in a cone, which in section appears as a wedge, with its apex projecting into the upper half of the blastula. In B (stage 7), the stain has reached the floor of the blastocoele, and in C is beginning to spread over the floor surface, first as a small circle of stained material, later, stage 8, in a radiating fashion or in a complete coverage of the floor, depending upon the intensity of original staining. During the latter part of stage 8 (D), there is a tendency to have a separation of the stain into a primary segment which passes toward the blastocoele cavity and a secondary aggregation which is drawn toward the presumptive yolk plug. This reaction is more marked in E (stage 9) and F (stage 10) when the folding over of the blastocoele floor and the formation of the underside of the gastrocoele floor begin to have a common cell boundary. The two movements, the primary and secondary, are still separate in stage 11, the stain of the primary being concentrated in the walls of the blastocoele and extending through the cellular boundary into the floor of the gastrocoele (G). In H (stage 12), the two areas of stain become confluent and remain so in later stages.

or pairs of forelimbs are developed. One develops from the normal somatopleuric mesoderm and the other from what would have gone into the formation of the splanchnopleure. These two sets of limbs, two rights and two lefts, are fully formed from each of the layers, but the splanchnopleure has not formed any of the smooth muscle which ordinarily would be present. There are visible, then, two definite defects associated with muscle formation, one in the region of the heart and one in the musculature of the gut. The stomodaeum is absent, as one would expect, and also the gills. The rest of the organism, however, is remarkably regular. These results change considerably our concept of how the muscle develops if we think conventionally of smooth musculature as being primitive, the cardiac as less primitive, and the somatic striate musculature as the most advanced. The facts seem to indicate a reverse of this sequence. The striate musculature is formed from the lateral mesoderm of the marginal zone. According to the findings of Vogt (1925, 1929), the areas of somite potency are well localized at the time when the marginal zone enters through the blastopore. It is at this time that it has its later characteristics impressed upon it.

The fact that the limb could be formed from splanchnopleure was already obvious if one considers the experiments of Harrison (1925) on the reversal of the mediolateral axis of the limb. Here, the limb was turned inside out, *i.e.*, the somatopleure and splanchnopleure were reversed with reference to the yolk. The splanchnopleure gave rise to perfectly good limbs and the somatopleure undoubtedly to good smooth musculature.

Nieuwkoop (*l.c.*), when he compressed the two sides of the material from which the yolk endoderm had been removed, showed cases in which he could distinguish the formation of a coelomic cavity which, however, had no splanchnopleure in association with it. It was entirely a somatopleuric cavity. He went also one step further and stretched the embryo after depriving it of its yolk upon a collodion membrane. In this case, development occurs in a manner strikingly reminiscent of a chick blastoderm, and again shows complete deficiency of all of the splanchnopleuric parts. No heart is formed except in those embryos in which the anterior parts had secondarily come together and even here the heart is markedly deficient.

The converse of these results is shown in Bacon's (1945) experiments. Bacon removed presumptive mesoderm from the outside of the embryo before the marginal zone had passed to the interior. When this marginal zone material was placed in contact with endoderm, it formed a heart rudiment. These heart rudiments, in some cases, were remarkably regular and were formed only after contact with the endoderm. There was no attempt to have the endoderm stay in relationship with the presumptive mesoderm, but they were dissociated after a period of contact, and yet remarkably normal hearts were secured, these hearts being kept entirely in isolation conditions. The fact that the tissue which ordinarily

would have developed into normal striate muscle forms a heart after having been placed in contact with endoderm shows definite inductive action of the endoderm upon the marginal zone material to form heart. Since the endoderm itself was taken from the general mass of material and was not localized, it seems as though there is a generalized influence extending through the endoderm which acts upon presumptive mesoderm for heart formation. It would be interesting to check this thesis in connection with certain definite spatially specific parts of the endoderm. This, so far, has not been done. It would also be interesting to try various sections of the marginal zone to see whether they would respond in exactly the same way to the various parts of the endoderm. An interesting series of experiments could be evolved in which these two structures were tried each against the other in order to test out the inductive effects. This induction can occur at a stage earlier than nervous system induction, thus placing the Spemann organizer in an entirely secondary role with regard to the formation of the embryo as compared to endodermal inductions.

Holtfreter (1944) has been studying the actions and reactions of various parts of the yolk endoderm. In many cases, he has restricted his observations to the cells which are coming in through the ventral lip of the blastopore. He finds that these cells, which form distinctive elements, are polarized and that when they are isolated in salt solution one can get at certain stages a centrifugal spreading of the non-polarized cells, and a definite polarization with an amoeboid process in the polarized cells. They have a proximo-distal axis which seems, to some extent, to be controlled in normal development by the action of associated cells upon the endodermal mass. The amoeboid process is generally extending away from the central mass of cells and Holtfreter has been able to show that, if four or five cells are placed together, they tend to have their proximo-distal axes arranged away from the center of the mass, the proximal portion being a rounded stub, while the distal part tends to be of the amoeboid type. Holtfreter likewise, in the same study, postulates that there is an independent movement in the cells of the endoderm, that this independent movement is responsible for a pulling of the structures so that they intrinsically occupy a definite mass and spread in a definite way. This, it seems, is endowing the endodermal cells with something a little more vital than one would expect, and probably more vital than the observational material would warrant. Whether the cells are pulling, or whether they have been displaced by the action of adjacent cells when acting in the normal organization of the embryo, is a question which still has to be decided. Holtfreter, however, has drawn our attention to an exceedingly important point in showing that, under conditions of isolation, the cells do certain definite things which seem to depend on mass aggregates and individual activity in response to that mass aggregate. No matter where the future of the endodermal situation will lead, these results will have a definite bearing upon our interpreta-

tion of how the floor of the gastrocoele is formed and what the effective forces are forming it. Certainly, the ventral lip of the blastopore has a much greater import in the organization of the entire venter of the embryo than we have hitherto supposed.

Kemp (1946) has changed various parts of the endoderm in relation to various other parts of the endoderm. He has removed sectors of the floor of the gastrocoele in the gastrular stage and has interchanged these either by cutting off one part of the embryo and then joining to it another embryo with a definite deficiency, or else by reversing, in some cases, the endodermal sectors in the lower portion of the neurula. These

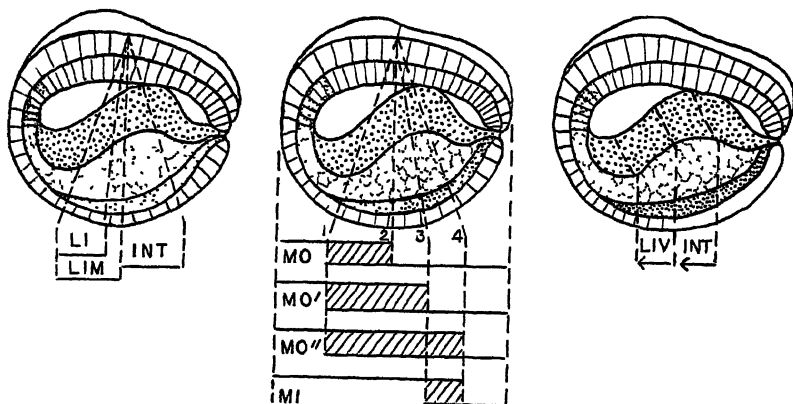


FIGURE 2. These three figures are redrawn from KEMP, 1946. The left figure shows the regions removed, comprising three series: Li, the anterior fourth of the mesenteron; Lim, the anterior third; and Int, middle third. The majority of the Li had functional tracts; Lim, non functional guts, all shortened, esophagus reduced or absent, liver and pancreas reduced, many heart defects; Int, 1 regulated completely, 92 per cent had functional tracts.

Middle figure—alteration of yolk endoderm arrangement by transection and reuniting of different levels resulting in duplication of parts of the mesenteron; MO, duplication of anterior third of mesenteron; MO', anterior half; MO'', anterior two-thirds; MI, post segment. MO—80 per cent functional, 48 per cent completely regulated, enlarged or double pancreas, duplicate gall bladders. (Table 2—KEMP.)

Right figure—shows the regions rotated to reverse A-P axis of the segment. The effects in this series are most marked after the reversal of the Liv segment as indicated by the percentage of abnormality produced. His conclusion is that each endodermal anlage is histologically determined but is equipotential within itself.

TABLE 1*
RESULTS OF DUPLICATION

Series	Number in series	Per cent showing complete regulation	Per cent with functional tracts	Per cent with duplicated pancreas	Per cent with duplicated liver
MO	25	48	80	20	0
MO'	35	5.5	23	62	6
MO''	32	0	25	100	6
MI	7	13	86	0	0

* From Kemp's Table 2, 1946.

have given rise to a set of rather interesting results, for in the course of this interchange he has been able to produce defects of the liver and of

the heart. His results are interesting in showing that a certain regulation can take place at this stage in spite of the way in which the endodermal material has been combined.

As mentioned above, Holtfreter considers that the endodermal cells are polarized at the time they pass through the blastoporal lip and that from then on the endodermal cells in the floor of the archenteron are effective in stretching and accommodating themselves to the region of the floor, passing out and forward as well as laterally, and pulling material from the posterior region into position. They are non-polarized before they enter the blastoporal lip. Their behavior with other cells when isolated shows this polarization complex even though the other cells around them are not reacting, *i.e.*, the cells which normally would encompass the entire mass and make up the dorsal lip and its lateral projections, the marginal zone with its involution; all these can be separated from the action of the endodermal cells in the floor of the archenteron. Holtfreter goes one step further in saying that the surface coat material which is pulled in with the ventral lip as well as with the dorsal lip acts in forming the linings of the cavities of the body as they are later found. This would seem to place a little too much emphasis upon the surface coat, which, although it comes in with the material, is strikingly reduced in the lower layer as compared with its amount on the outside of the egg itself.

Holtfreter (1933) gave one of the best examples of what can happen in his exo-gastrulation studies. In this case, after having treated the embryos with hypertonic solutions, he secured complete exo-gastrulation with the mesoderm forming its parts lying on top of the endoderm and forming gill arches and body form without the action of the ectoderm. This is an interesting check on the results which Nieuwkoop obtained, for here we have the situation exactly in reverse. The Spemann organizer has not acted in forming the nervous system. Instead, however, during exo-gastrulation, the mesodermal parts have been acted upon, forming a perfectly good head region without, of course, the nervous system, forming gill arches, and forming the body which has the general conformation of what it would have had at the same stage if the embryo had developed with the ectodermal covering around it. The ectoderm, potentially capable of making nervous system as well as body covering, has formed only a bladder of material in which there is no differentiation except that of a common epidermis.

In his 1938 and 1939 studies, Holtfreter has performed other experiments showing still another part of the reaction of the endoderm. When ectoderm and mesoderm are united, a vesicle is formed containing connective tissue, but little else. When the endoderm is added to the isolated bladder of material, there is definite structural conformation and arrangement of the mesoderm, the formation of certain muscular parts in connection with the endoderm, the whole being surrounded by ectoderm. The ectoderm in this situation does not influence the mesoderm nearly

so much as does the endoderm, for in the endodermal parts we find a definite uniting of structure, with the mesoderm contributing a muscle layer and the outside sheet of the gut which is differentiating from the endoderm. Here, the potentiality of the gut region is clearly marked; it has a much greater definitive potency for calling forth from mesoderm a differentiation unlike the connective tissue which would develop within the ectodermal bladder by itself. This is another demonstration of the effective facility with which the endoderm acts upon associated structures.

The experiments of Nieuwkoop and Holtfreter strikingly demonstrate that, in the gut endoderm, we have certain capacities for organization which have been neglected in looking upon the embryo as a whole. Its capacity to organize by induction, the facts that the heart is missing without the influence of the yolk endoderm and that the gills are completely missing, had been noted in Stöhr's (1931) experiments. He suspected that the endoderm exerted some influence upon the reacting structures in causing the formation of heart. He did not, however, suppose that the endoderm was directly responsible for smooth musculature of the gut and that the somatopleure and splanchnopleure together could form interchangeable structures.

It is interesting, therefore, to review the morphogenesis of the muscular system, for the splanchnic mesoderm, if not in contact with endoderm, will form striate muscle under the influence of the somatopleure. Any part of the marginal zone which is potent to form muscle striate in character can be induced, according to Bacon's results, to form heart in the presence of endoderm. The reversal of the limb bud (placing the splanchnopleure on the outside, the somatopleure on the inside) gives rise to a perfect limb. All these things tend to show that there is a dominant action exerted by the endoderm which has a part in the induction of muscular structure. It also shows that the final determination of such structure lies not only in its general position, but also in its definitive relationship to other tissue influences.

In the light of Holtfreter's (1939) experiments, it was thought advisable to carry the isolation of the yolk back further into the foregrounds of embryonic history than he had done. Yolk endoderm of the late blastula, when grown in culture (Nicholas, 1945), assumes about the same form relationship as would be found in the normal animal. When isolation experiments were performed upon young blastulae, it was found that not enough of the plasmagel of the external coat material was present in the endoderm to permit its attainment of form. The experiments in which ectoderm is combined with yolk endoderm, as shown in FIGURES 3 to 8, are more successful when different proportional amounts of ectodermal-endodermal material were used. When yolk endoderm material is taken from the early blastula and cultivated without an external cover, the results are rather clearly shown (FIGURE 4). The cells aggregate and so does the nuclear and pigment material; there is a fine granular

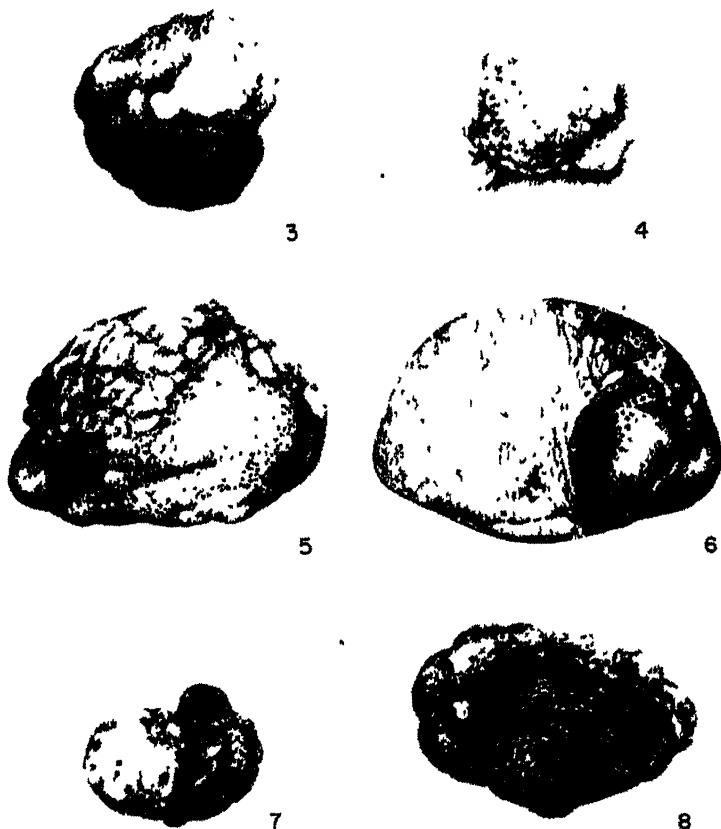


FIGURE 3. Isolation study of yolk endoderm from early blastula enclosed in envelope of ectoderm. Cultured 5 days. A thin coat covers all of the exposed endodermal material. The ratio of endodermal material to ectodermal cover is 2 to 3.

FIGURE 4. Yolk endoderm of late blastula cultured in the absence of ectoderm in double Holtfreter's solution. Note the cell aggregate and the clumping of pigment and nucleus within the constituent cells. Cultured 1 day. There is no ectoderm present and fine granular material was being exuded from the loose cells located in upper part of the diagram.

FIGURE 5. Yolk endoderm isolate, mid-blastula, in combination with a small ectodermal piece, shown to the left of the figure. The ratio of endoderm to ectoderm is 5 to 1. After 5 days of culture, disintegrative action began, as shown by the loose cells in the upper left of the figure.

FIGURE 6. Yolk endoderm isolated in early blastula and combined with ectoderm in 4-to-1 ratio. There is here, as in the previous figure, a distinct antero-posterior lengthening of the yolk endoderm. There is a rolling of the cells at the inferior margin of the figure, showing that movement of the endodermal constituents continued under these conditions.

FIGURE 7. A 1-to-1 combination of yolk endoderm of an early blastular fragment with ectoderm from the blastular cap. After 7 days of culture, there is no axial or other differentiation, probably due to the small size of the piece.

FIGURE 8. Isolate consisting of 1 part of yolk endoderm combined with 5 parts of ectoderm. The ectoderm contains a small ball of endodermal material which in 6 days of culture did not differentiate.

type of exudate, probably made up of the yolk platelet content, and in spite of the fact that the isolates were kept in double Holtfreter solution, which is osmotically favorable to endoderm preservation, they disintegrate usually within 24 hours. If, however, ectoderm is combined with the yolk endoderm, as shown in FIGURE 3, there is a tendency for the endoderm to receive something from the ectoderm which acts as an external cover preventing the disintegration of both the ectoderm and the endoderm.

The same relationship is shown in FIGURES 5 and 6, which are examples of about the minimum of ectoderm combination which is necessary for the development of the yolk endoderm taken from the early blastula stages. FIGURE 5 shows rather interestingly that the external cover over the yolk endoderm is not adequate to prevent sloughage, which can be seen in the large, loose, white mass of cells to the left of the figure. An isolate (FIGURE 6) in which there was about 25 per cent of ectoderm at the time of its first isolation, has a complete coverage and can be maintained for a considerable period of time. The elongation of the yolk endoderm under these conditions simulates the normal axial relationship. The ectodermal covering is found near the anterior end of one of these masses and near the posterior end of the other, as judged by their general morphological appearance. Ectoderm by itself does not seem to have a definite effect upon the polarization of the yolk mass. An early isolate in about the 128-cell stage is illustrated. Here, an increasing amount of ectodermal material is necessary if the isolate is to survive in double Holtfreter solution. As in previous figures, one sees the relationship of the ectoderm to the yolk endoderm in that there is a consistent smooth covering of material on the outside of the endodermal cells which they themselves do not produce in amounts adequate to cover their surface. In FIGURE 8, also an isolate, in which much more ectoderm was taken and where the relative amount of the endoderm was small, the ectoderm completely covers the endoderm, the endoderm remaining a small, nodular mass inside the ectoderm. While it retains its normal components, there is no indication that there is any development of polarity of axial determination.

In this series, then, it is shown that material from the early blastula lacks the capacity for the formation of a superficial plasmagel in sufficient quantity to prevent content losses to the surrounding medium (in this case a double Holtfreter solution).

When varying proportions of ectoderm, taken from any region of the blastula, are included with the early blastular endoderm in the isolation, the endoderm can be maintained without disintegration. The self-differentiating powers of the yolk endoderm cannot be demonstrated unless it secures additional material from potential ectoderm cells which increase its surface membrane and enable the endoderm to undergo its own arrangement.

This is not regarded as a direct morphogenetic effect upon the en-

doderm by the ectoderm, but as a mechanical factor supplied by this material in the form of plasmagel. Elongation and the expression of axial tendencies are obtained only when the yolk endoderm of the early blastula is present in adequate proportions. A four-endoderm-to-one-ectoderm combination, as shown in FIGURE 6, is optimal. When the proportions are reversed, as in FIGURE 8, one endoderm to four ectoderms, the endoderm shows no elongation but remains as a rounded ball with an ectodermal cover.

A separate series of observations was made upon early blastulae in which the roof of the blastocoele was removed, and a piece of the transparent vitelline membrane was cut to fit the opening and inserted so that the ectoderm was fitted around the margins of the cover. It was possible, by this method, to observe the formation of the floor of the blastocoele and the way in which the various parts came into their organization complex during the course of development. The vitelline membrane remains transparent, is retained by the organism without apparent damage to the adjacent tissues, and, since it softens during development, serves to separate the various parts during the critical period in the organization of the floor of the archenteron. It was noted early by this observational method that there was a pattern of cells arising near the midpoint of the blastula in the floor of the blastocoele. There are generally between 16 and 28 of these rather large cells, which appear in the form of the mosaic in the floor of the blastocoele. These are the cells which have formed in the pathway of ingression (see FIGURE 1). Whether they are actually cells that were originally on the outside cannot be definitely stated. There are probably many more cells, not distinctive in either their form or their position, which come in at this time, and certainly the 16 to 24 which projected the pebbled surface on the floor of the blastocoele can be localized as being morphologically in the chain of ingressive material. They later are found under the stomodaeal pit as the blastocoele becomes reduced.

In addition to the experiments reported above, in which the observations of the cells in the floor of the blastocoele could be made, additional experiments were conducted in which the entire cover of the early gastrula was removed. In this series of experiments, the neural folds developed at the marginal limits of the denuded area; the yolk endoderm was exposed, and the results of the previous series of experiments were checked. Just anterior to the midpoint of the blastocoele floor, the pebbled series arises, forming a portion of the floor of the archenteron. Above this develops the stomodaeal pit, while posteriorly the proctodaeal pit is found. These two structures seem to develop entirely from the yolk endoderm, which is specific for them. The factors which have to do with the formation of the stomodaeal pit and the proctodaeal pit certainly have their origin intrinsically in the yolk endoderm. The occurrence of the lateral neural folds presented somewhat of a problem, since this material was underlain only with marginal zone material which nor-

mally would have become lateral mesoderm. When the substrate is marginal zone material, however, and not the usual chorda mesoderm, it has the capacity and competence for forming ectoderm into nervous tissue (see FIGURE 10). The neural folds, while poorly developed anteriorly,

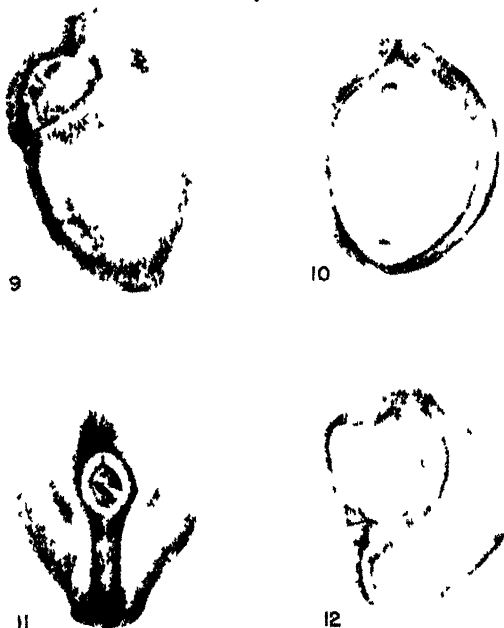


FIGURE 9. The utilization of the transparent vitelline membrane as a blister through which observations upon the formation of the stomodaeal invagination are made. The neural folds, are held apart in the region of the mid- and hindbrain.

FIGURE 10. A culture of the yolk endoderm held in place by the ventral ectoderm and the marginal zone. The operation removed practically the upper half of the blastula. The formation of the proctodaeal and stomodaeal invagination is regular and occurs because of reactions intrinsic to the ventral half of the early blastula.

FIGURE 11. A small cap was removed from the dorsal part of the blastula and observations made upon the midpoint of the blastocoele floor (cf. FIGURE 1). The cellular mosaic developed regularly in the floor and is later, by the involution of the blastocoele, converted into the floor of the archenteron beneath the stomodaeal groove.

FIGURE 12. Culture of early blastula with same type of operation as shown in FIGURE 11 but without vitelline cover. The growth processes in the endoderm are less regular and a part of the endoderm has been displaced in the healing process.

show good structural relationships, and the sections through these in individuals show perfectly definite systems.

If the experiment is performed later, only part of the roof of the mid-gastrula is removed and the cellular reactions which are going on in the floor of the gastrocoele can be observed. The nervous system, instead of consisting of half the nervous system in the regions which have been

separated by the removal of the roof of the blastula, now is practically complete.

There is a distinct difference, then, in the nervous system effect of the two operations, for in the first case the nervous system forms really as a vestigial type of regenerate; in the second case, where only a small portion of the roof of the blastula was removed, it is practically complete.

Discussion

The question immediately before us to be considered is not that of the localization of the parts of the gut in the yolk endoderm. This is adequately treated in Kemp's (1946) paper. In general, the results given here support Holtfreter's (1939) conclusion that there is early localization of the gut. The stomodaeum and proctodeum are determined in the late blastula, and there are indications that the morphological components which determine this change are the elements which have reached the inside of the blastocoele by ingression. This is a rather important point, for it shows an early localization of gut structure as well as an antero-posterior localization of the materials. The parts, however, between the stomodaeum and the proctodeum seem to have only a general relationship.

The general relationships of the yolk endoderm are more important for the present discussion than the absolute localization of parts such as liver, or the parts of the liver. As has been pointed out previously, the yolk endoderm has a generalized capacity which can call forth from the surrounding tissues certain definite elements. This is also clear from Nieuwkoop's experiments, for, in his study of the origin of the germ cells, one can see the various ancillary actions which the yolk endoderm exerts. It is positive in the formation of the stomodaeum and the heart which shows from Stöhr's experiments (which are the converse of Bacon's), as well as in various experiments having to do with the gills and their formation. The endoderm, then, is exceedingly potent in the formation of structures at the anterior and ventral aspects of the embryo. The fact that splanchnopleure does not develop if the gut is absent shows a definite directive effect of the yolk endoderm upon the histogenesis of what might be regarded as a generalized muscular structure. The inhibiting action of the yolk endoderm which prevents the splanchnopleure from forming limbs is another important indication of its action.

Yolk endoderm is probably the largest source of the chemical materials which will be transformed by the organism. These are the rough products from which the later chemical materials are going to be formed. It is possible that the yolk platelets which have been separated into the ectodermal cells and which are part of their composition are changed in their values with relationship to the chemical constituents by the cells themselves. If this is so, we have a rather clear indication that certain of the materials which are found predominantly in the

nervous system later are developing from the raw materials of the yolk endoderm which have been carried about and placed in a definite location by the cellular activity itself. The greater reservoir of these materials still remains in the yolk endoderm which is left behind.

In watching the development of the amphibian forms, one is frequently impressed with the fact that the material found within the gut, consisting of the yolk endoderm particles, which have been enclosed by the gut material, does not completely digest, and, while there is an extraction of material from the yolk, there is still sufficient residue left to make quite a sizable collection of material. This material is relatively inert and should be investigated in order to get the difference between the chemical substances which are involved in the yolk platelet formation and the final residue after its extraction by the organism. This should form a field of rather useful investigation for the chemical understanding of what has gone into the organic relationships which have been formed by the embryo itself.

The yolk endoderm, then, has been shown in the present discussion to be responsible for the formation of the stomodaeum, to take part and be active in the formation of gills, and to possess the capacity for induction of the heart from indifferent mesoderm or from mesoderm which is prospectively significant for the formation of other structures than the heart. Since the yolk endoderm is so active in the formation of anterior structures, it should be looked upon as a possible factor influencing the formation of both the hypophysis and the thyroid. It has a corollary in Nieuwkoop's findings that the endoderm is responsible for the induction of germ cells from the lateral plate. From Nieuwkoop's work, it is clearly shown that the muscle developing from the somites in the splanchnopleuric region will not develop as the smooth muscle of the gut, but acts in the absence of the yolk endoderm as somatopleure which has the capacity for developing limbs at the normal regional level similar to that found in the somatopleure. When the yolk endoderm is removed in early stages, the heart is atypical or absent. When the yolk endoderm is cultured in mid-blastula stages, it elongates, possesses a definite plasmagel coat, and develops the invaginations normal to the formation of a stomodaeum and proctodaeum. In earlier stages of the blastula, some material from the outside of the egg must be incorporated along with the yolk endoderm in order to maintain it as a unit and have it continue its development. One can secure the development of practically normal form and extension if the yolk endoderm is 25 per cent of the quantity of the graft and if it is covered by external material. This points to the necessity for the normal operation on the yolk endoderm of material from the outside of the egg. The fact that the mid-blastula stages can be cultured shows that sufficient material of a nature similar to that of the external coat of Holtfreter is present in the cells of the yolk endoderm and can take care of its relationship with the external environment.

The development of the chemical substances and the enzymes which are effective in energy transformation may occur in the cells that carry a maximal or minimal amount of the yolk platelets. These are particularly rich in the endodermal cells, as is shown by the cytochrome oxidase and probably by the adenyl-pyrophosphatase development.

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THE ROLE OF NERVES IN AMPHIBIAN LIMB REGENERATION*

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THE first study of the influence of nerves on regeneration was reported by Tweedy John Todd in 1823.⁶⁰ He had discovered that division of the sciatic nerve in the limb of a salamander prevents regeneration distal to the level of division of the nerve. He further stated that division of the nerve causes a regenerate already developing to shrivel and waste. These observations lay unused for many years, until interest revived and Todd's paper was rediscovered early in this century. Several papers appeared soon after the year 1900, some affirming and others expressing doubt^{9, 37, 65} that nerves are necessary during regeneration. The question was finally settled in the affirmative by Wolff in 1910⁶⁶ and by Walter in 1912.⁶⁴ Later, Schotté made it clear that if a nerve supply to a salamander's limb is interrupted, regeneration does not begin until after nerves have regenerated and re-entered the region adjacent to the level of amputation.⁴⁴ The expressions of doubt had apparently been due to faulty operations in some cases and to failure to recognize regenerated unmyelinated fibers in others.

Prior to the analysis of the role of nerves in regeneration, attempts were made to isolate the various components, sensory, motor and sympathetic, and to determine thereby which are necessary for regeneration. At first, it seemed that only the sensory supply was necessary because regeneration could occur in the absence of the section of the spinal cord supplying the limb plexus if the dorsal root ganglia and the sensory fibers were not severed.⁶⁴ This was regeneration in the absence of a motor supply. Schotté reinvestigated the question of which nerve component, motor or sensory, is needed and found inconsistencies in his results. For example, sometimes the sensory supply alone seemed to be enough for regeneration, at other times a regenerated motor supply was sufficient, and even the original motor supply seemed to be enough in a few cases.⁴¹ It was suspected that the inconsistent failures in regeneration were caused by chance interruption of the sympathetic nerve fibers.⁴² Following this hypothesis, attempts were made to eliminate the sympathetic supply in some cases, and in others to remove everything but the sympathetic. The results were never completely consistent, but numbers favored the sympathetic nerves as the important component for limb regeneration.⁴³

This conclusion was widely accepted, but it did not go wholly unchallenged. Locatelli pointed out that the sympathetic nerves, especially

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the communicating rami, are extremely small, and the rami, at least, cannot be seen through the dissecting microscope.²⁰ She refused to accept the conclusion that the sympathetic component is important in regeneration, because of the extreme difficulty of the operations and the failure to confirm them histologically. Her own experiments reaffirmed that limbs can regenerate with sensory fibers alone. Working with the nerves supplying the hind limbs, she dissected around the roots of nerves 17 and 18 in order to remove communicating rami and then pulled 17 and 18 free from the cord. The operation was done in such a way that both ventral and dorsal roots were torn, but the dorsal roots were torn proximal to their ganglia. This left the sensory fibers of the limb still attached to their cell bodies but severed the connections between motor fibers and their cell bodies in the cord. With only the sensory fibers intact, regeneration was normal. In some cases, the sensory component of the 18th nerve by itself supported normal regeneration, but there was no regeneration if the 18th ganglion was extirpated. These results were taken to mean that the 18th dorsal root ganglion had a regeneration-promoting quality peculiar to itself.²⁰

This was the state of the problem in 1929. Doubt had been cast on the claims for the sympathetic, and only part of the sensory supply seemed necessary for limb regeneration. In 1942, Singer began to publish an experimental re-analysis of the problem. His first finding was that post-ganglionic sympathetic fibers passing to the anterior limb in *Triturus* do not join the mixed nerves of the brachial plexus by way of communicating rami. Instead, the post-ganglionic fibers leave the sympathetic cord and constitute a separate nerve, the subclavian, closely applied to the subclavian artery.⁵² Older observations (see Singer, 1942a, for bibliography) show a subclavian nerve to be the usual pathway for sympathetic fibers leading to an anterior limb in Urodeles. This fact invalidates the earlier experiments on the sympathetics. A reinvestigation showed that complete unilateral sympathectomy in the anterior region did not prevent normal regeneration on the side concerned.⁵³ In addition, there are reports that forelimbs had regenerated normally after their subclavian arteries had been severed.^{14, 44, 67} Since it would be extremely difficult to sever the subclavian artery without also severing the nerve, these observations lend weight to the conclusion that limbs may regenerate without sympathetic nerves.

Further careful re-analysis by Singer, with operations checked histologically, show the truth of the thesis that the sensory is the only component which by itself can support normal regeneration.^{53, 54, 55} However, this does not mean that sensory neurones have a peculiar quality enabling them, and them alone, to support regeneration. This is not true, as Singer shows, for, although a normal motor supply will not support regeneration,⁵⁵ a regenerated motor supply will.⁵⁶ The key to an understanding of this paradox comes from a consideration of the number of fibers involved. There are many more motor fibers after regenera-

tion from a ventral root because the axones branch repeatedly as they regenerate. It has been determined that the number of fibers necessary for limb regeneration in *Triturus* is in the range of one-third to one-half of the total number.⁵⁷ The normal motor component does not contain a third of the total number of fibers, but there are enough fibers in a regenerated motor supply to satisfy the threshold requirements. The sensory component is the only normal component with enough fibers to promote regeneration by itself. In fact, there are so many sensory fibers that not all of them are needed. Unless the assumption is made that the regenerated motor fibers have acquired a new quality not present in the normal motor supply, it seems likely that neurones of all components possess the quality necessary for regeneration. Certainly, sensory and regenerated motor nerves do.

In the course of her experiments, Locatelli discovered that limb nerves deviated from their normal course and, when made to end near the base of a limb, stimulated the development of a new limb over the end of the nerve.¹⁹ The idea that nerves possess specific morphogenetic potency was suggested, but had to be discarded after a demonstration that limb nerves deviated to adjacent territories stimulated regeneration of structures whose form was determined by the territory rather than by the nerves.^{11, 2} For example, when the limb nerve ended at the base of the dorsal crest, a piece of dorsal crest developed.

We now face the problem: How do nerves stimulate regeneration? In trying to discover their function, we are aided by the knowledge that embryonic limb buds can grow and differentiate without a nerve supply, as first demonstrated by Harrison.^{13, 12} Since nerves are not necessary in embryonic development, it would seem that our search should begin with that phase of regeneration which differs from embryonic development. That phase is the first phase in regeneration, really a preparatory one, during which the old tissue organization at the level of amputation is lost, and free cells, apparently released from old tissues, collect and form a bud.^{3, 45, 21, 62, 31, 28, 6, 59} This change from organization as tissues to a cellular type of organization is known as dedifferentiation. The bud or blastema, once it is established, resembles an embryonic limb bud, and from that time on its development is similar to the development of an embryonic limb. It is here, during the preparation of the blastema, that we shall first look for an influence of nerves.

The fact that nerves are required during this early phase of regeneration is clear: a blastema does not form in the absence of nerves. Only lately has there been much work on the causes of failure to regenerate after denervation. Schotté⁴⁴ and, later, Butler and Schotté⁵ and their co-workers^{50, 51} demonstrated that denervated limbs of young, larval salamanders begin to dedifferentiate in the region of an amputation surface but fail to regenerate because dedifferentiation and resorption continue unchecked until whole limbs disappear. The reason for failure to develop

a blastema is quite different in older limbs and it is with them that we shall begin our analysis.

It is necessary to digress for a moment in order to introduce a regenerative process which we shall show to be influenced by nerves. It has been known for a long time that there is no regeneration from a wound sealed with skin.^{61, 58, 8} This is not the result of a physical block to blastema growth. What a complete skin seal does is this: By covering a wound, it prevents an epidermal epithelium from spreading over and into the wound, thus barring close contact of epidermal wound epithelium with underlying tissues. In normal regeneration, it is the close contact of wound epithelium with underlying tissues which causes histolysis of those internal tissues and the release of free cells as shown by Jeffmoff^{15, 16} and by Polejaiev.²³ A complete skin over a wound prevents regeneration, not by preventing growth of a blastema—far from it—but because it prevents dedifferentiation which provides the cells for the blastema. If a limb wound is covered in such a way that one corner of a skin seal is free, a wound epithelium can migrate in under the skin covering the wound and do its work.¹⁵ Intense internal histolysis leading to blastema formation results and, instead of the skin acting as a physical barrier to growth, it, too, undergoes histolysis.⁸ Not all epidermal wound coverings, even in salamanders, cause extensive dedifferentiation of the tissues underlying them. For example, although epithelia from limb, tail or abdomen will support limb regeneration, wound epithelia provided by skin of the head or back will not.^{16, 29}

Passing, now, to a non-regenerating animal, the adult frog, we find that one of the causes for failure to regenerate is a change in the epidermis during metamorphosis.^{24, 49} Miss Gidge and I obtained good regeneration of forearms and wrists in adult frogs after substituting tadpole skin for their own adult skin.⁷ A wound epithelium provided by the transplanted tadpole skin migrated over and into the wound of amputation and initiated extensive dedifferentiation. This is very limited in adult frogs if their own epidermis contributes the wound epithelium.³⁶ The heavy dermis of adult frogs may also be a factor in preventing regeneration because it, along with epidermis, soon closes over a wound, forming a barrier between epidermis and underlying tissues.³³ However, adult anuran limb epithelium can be stimulated by treatments with NaCl^{32, 33, 35} or with other irritants^{26, 27} and will then act as a younger epithelium and, in its turn, cause dedifferentiation even of dermis with the concurrent release of cells to be used in a blastema. FIGURE 1 is a section of a salt-treated frog limb. If it is compared with FIGURE 2, a control limb of the same age, it will be seen that dedifferentiation had just started in the salted limb. Bone, for example, had begun to erode away. Comparison of a salt-treated limb (FIGURE 3) and a control limb (FIGURE 4) a few days later shows intense dedifferentiation and concurrent blastema formation in the salt-treated limb while the control has already

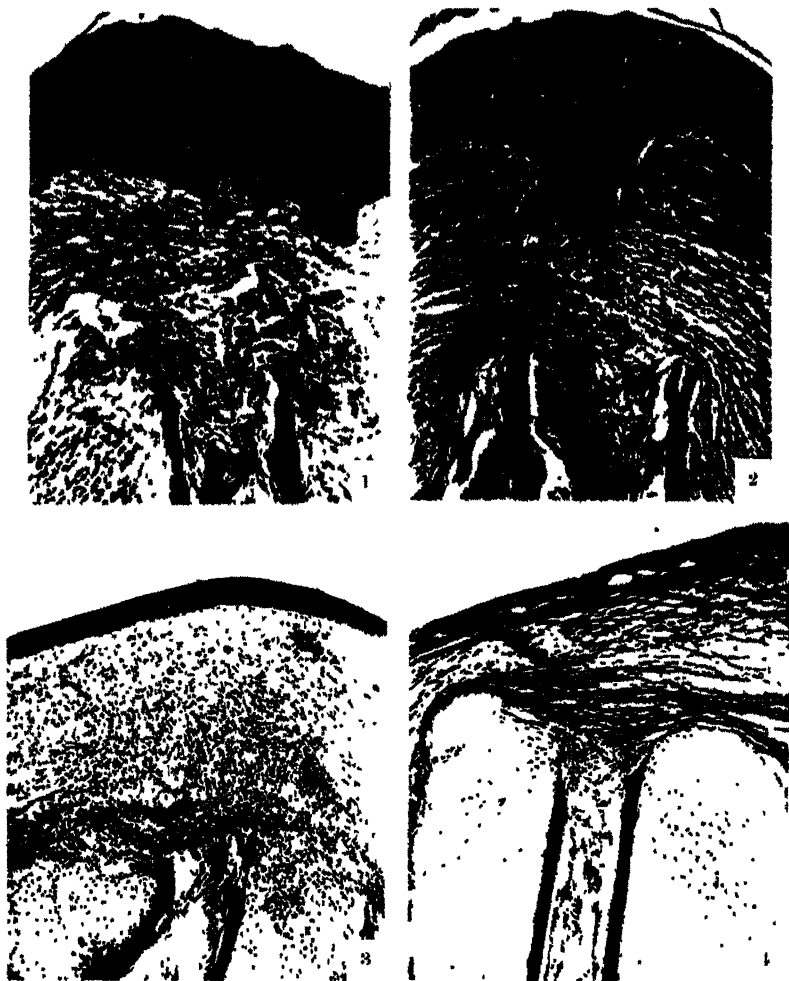


FIGURE 1. A longitudinal section through the tip of a salt-treated frog's limb 19 days after amputation and 10 days after the last salt treatment. Internal dedifferentiation is just beginning. Erosion of bony matrix and the presence of several large, darkly stained, free-fused osteocytes may be noted. X70.

FIGURE 2. A longitudinal section through the tip of an untreated frog's limb 19 days after amputation. This shows no active dedifferentiation and is to be contrasted with the salt-treated limb in FIGURE 1. X70.

FIGURE 3. A longitudinal section through the tip of a salt-treated frog's limb 27 days after amputation and 18 days after the last treatment. A large blastema of mesenchymal cells has formed, beneath which is a zone of dedifferentiating old bone and new cartilage. X45.

FIGURE 4. A longitudinal section through the tip of an untreated frog's limb 27 days after amputation. Already, fibrous scar tissue and cartilage, flanking the bone, have completely differentiated. This premature differentiation is in marked contrast to the undifferentiated state seen in FIGURE 3. X45.

healed with a cartilaginous callus surrounded by fibrous scar tissue. The dedifferentiation had apparently been initiated by a stimulated epithelium.

The best of the regenerates obtained from adult frogs by either salt treatments or tadpole skin transplantation, after amputation through the forearm, had normal new forearms and wrists but were abnormal in the more distal regions (FIGURE 5). We shall consider the cause of this abnormality later. At present, we are interested in salt treatments only because of their action in stimulating a wound epithelium.

Returning from the digression with the knowledge that an active wound epithelium causes internal histolysis, we can now show that nerves are normally involved in the activation of the wound epithelium in adult salamanders, as Miss Oliver and I²² have recently demonstrated. FIGURE 6 shows a blastema on a normal limb of the newt, *Triturus viridescens*. Contrasted with this is the tip of a non-regenerating denervated stump of approximately the same age (FIGURE 7). In the denervated limb, there has been very little dedifferentiation. Only the cut muscle fibers have degenerated. Bone, a good landmark, remains intact to the end of the stump. This stump failed to develop a blastema in the absence of nerves because it failed to dedifferentiate. Notice the wound epithelium, only a few cells thick and composed almost entirely of squamous cells. Now compare a completely denervated limb of the same age which had received baths in a strong NaCl solution (FIGURE 8). The wound epithelium is much thicker and extends down into the wound. This time, there is active dedifferentiation. Bony matrix is being eroded away and, in every case, multi-nucleate, fused osteocytes lie in the wake of the process. The conclusion is drawn that nerves promote dedifferentiation in the adult limb stump.

Dedifferentiation is not the only process for which nerves are needed during regeneration, as we learn from consideration of the ultimate fate of the limbs in question. Cells in the blastemata of normal limbs grow rapidly and remain undifferentiated until a large group of cells has formed. Only then does differentiation begin. In the denervated, salt-treated limbs, there is appreciable dedifferentiation, but the most striking phenomenon is the rapid differentiation of new tissue, cartilage, and fibrous scar tissue, with a concurrent failure of growth. In the untreated, denervated limbs, few cells dedifferentiate, but the few which do so almost immediately redifferentiate again into scar tissue and cartilage. In FIGURE 9, showing a denervated limb of the same age as those in FIGURES 7 and 8, premature differentiation of cartilage along the edge of the partially eroded bone may be seen. This is never observed in salamander limbs with a normal nerve supply. It would seem that nerves promote growth without differentiation, or, rather, shift the balance between growth and differentiation toward growth. In this way, a sufficiently large mass of tissue is available to form a limb by the time differentiation begins.

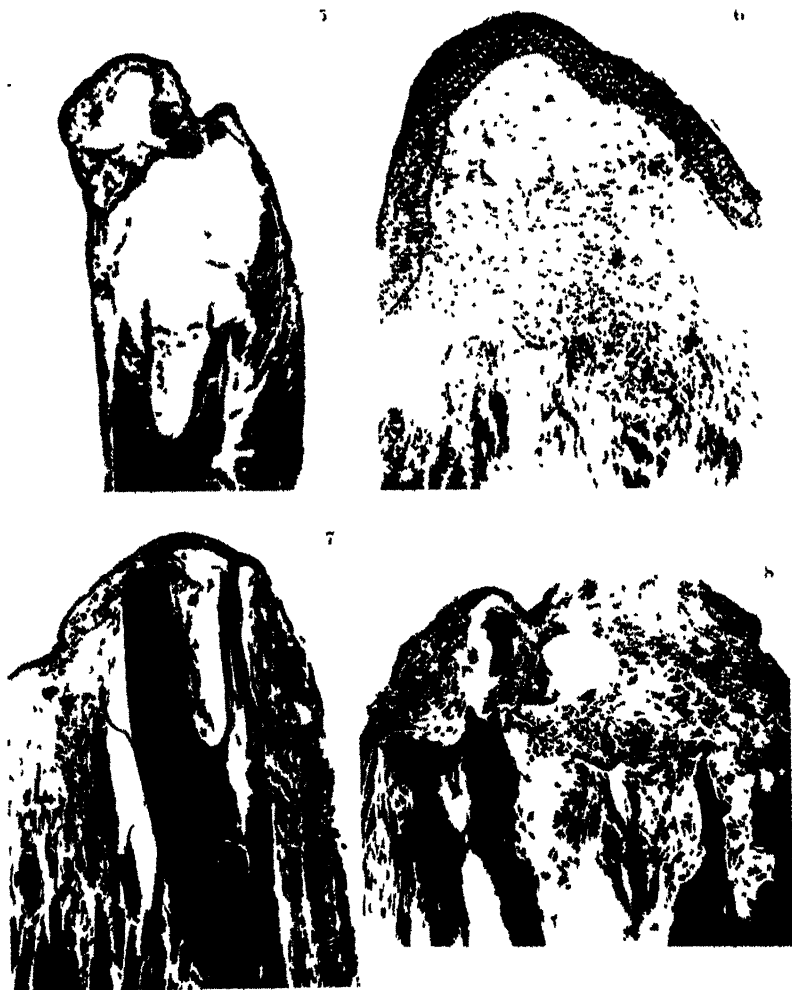


FIGURE 5. One of the best of the frog limb regenerates obtained after transplantation of tadpole skin. The limb had been amputated through the middle of the forearm. Pictured here is the regenerated distal forearm and normal functional wrist. It is to be noted that in this and in all of the induced regenerates the distal part of the hand and the fingers are either abnormal or missing. X12.

FIGURE 6. A cone-stage blastema on the forearm of an adult *Triturus* 23 days after amputation. Dedifferentiation had progressed as far as the tissues at the base of the photograph, and rapid growth of the blastema had begun. X70.

FIGURE 7. The tip of an adult *Triturus* forearm 23 days after complete denervation and amputation. There has been very little loss of structure. Cut muscle fibers have degenerated, but bone is still intact even at the level of amputation. X70.

FIGURE 8. The tip of a salt-treated adult *Triturus* forearm 23 days after complete denervation and amputation. The limb had been treated with strong NaCl solutions for the first 14 days after the operation. The greatly thickened wound epithelium, typical for treated limbs, is to be contrasted with the thinner wound epithelium on the untreated limb in FIGURE 7. Dedifferentiation of bone had begun in the treated limb. X70.

There is supporting evidence for this belief. If a denervated newt limb is re-amputated after 23 days, some nerve fibers have apparently re-entered the limb and a small fraction of these fresh stumps will regenerate limbs, but most of them are insufficiently innervated. These produce callus and scar tissue, as seen in FIGURE 10. The same type of growth was observed by Walter many years ago⁶⁴; it was also caused by insufficient innervation. There can be little doubt that nerves are important for limb regeneration not only because they favor dedifferentiation but also because they promote rapid growth of the dedifferentiated cells. If growth fails and differentiation begins prematurely, all the cells in the vicinity of bone are transformed to chondrocytes and others closer to the periphery become fibroblasts.

This combination, inadequate dedifferentiation followed by poor growth and rapid dedifferentiation ending with callus formation, is found not only in denervated and x-rayed limbs, but also in the limbs of naturally non-regenerating vertebrates.^{34, 35} FIGURE 11 is a section through a 9-day stump of a frog's limb. Already, its limited dedifferentiation and premature differentiation have resulted in the formation of callus and scar. The same picture is seen in a lizard limb stump (FIGURE 12). Here, too, fiber and cartilage form prematurely, sooner than these same tissues form in the regenerating tail of a lizard, as shown by Barber.¹

The question naturally arises: Do these limbs fail to regenerate because of insufficient innervation? An answer in the affirmative might be closer to the truth if the question were worded: Have the limb tissues of the non-regenerating vertebrates evolved in such a way that the work which nerves do has become more difficult? Changes which occur in anuran tissues at metamorphosis and which cause failure to regenerate are reviewed in an excellent article by Polejaiev in the *Biological Reviews* for 1946.²⁸ It might be possible to overcome these changes in some of the non-regenerating forms by increasing the quantity of nerve fibers in the limb. There has already been initial success along this line. Singer reported in a discussion, late in 1946, at the A.A.A.S. meetings in Boston that he had stimulated partial limb regeneration in adult frogs by deviating the sciatic nerve to the forelimb. We are awaiting the results of our own attempts to stimulate limb regeneration in lizards by increasing the amount of neural tissue.

Returning again to the salamanders, we find that the need for nerves is not over as soon as a blastema has formed, as demonstrated by Weiss,⁶⁷ Schotté,^{44, 46, 47} and Samarajew.^{38, 39} If nerves are sectioned in a salamander limb during the early stages of blastema formation, the blastema regresses. Even after a regenerate has advanced to the stage when the first form changes are apparent, interference with the nerve supply may prevent the formation of distal structures or cause them to be abnormal.^{44, 38, 39} The structures affected are the last to take form. Their formation is ordinarily accompanied by rapid growth. When the nerves

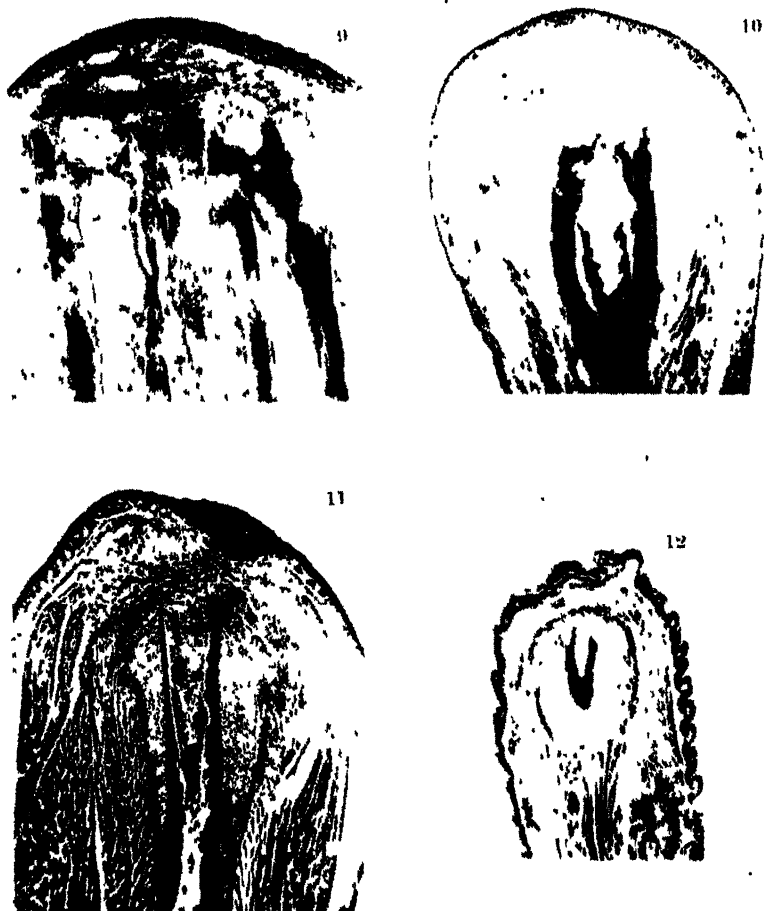


FIGURE 9. An untreated *Triturus* limb 23 days after denervation and amputation. In this denervated limb, there had been slight erosion of bony matrix, but this was almost immediately followed by differentiation of cartilage, which may be seen at the extreme right of the photograph along the shaft of the bone. There is another small island of differentiating cartilage to the left and near the middle of the bone on the left. X70.

FIGURE 10. An untreated *Triturus* limb 4 months after a reamputation which had been performed 23 days after the original amputation and denervation. This limb, which probably had some regenerated nerve fibers at the time of reamputation, has formed a cartilaginous callus covered by fibrous scar tissue. X35.

FIGURE 11. A normal untreated stump of a frog's forearm. Here, as in FIGURE 10, can be seen the cartilaginous callus and the fibrous scar tissue. X22.

FIGURE 12. A normal untreated stump of a lizard forearm. This naturally non-regenerating limb shows the same arrangement of cartilaginous callus and scar tissue as seen in the non-regenerating frog's limb (FIGURE 11) and in the non-regenerating partially innervated newt limb (FIGURE 10). X12.

are sectioned, the growth rate decreases markedly. It seems likely that these distal abnormalities result from failure of growth at this critical

period. Differentiation unaccompanied by rapid growth also seems to be the cause of distal abnormalities in induced anuran regenerates.

The various treatments so far used to artificially stimulate regeneration have provided large amounts of dedifferentiated material but have not increased growth during the period of finger formation when growth is so necessary a part of morphogenesis. Again, we see the adult anuran limb failing in a process which is supported in the urodeles by nerves.

As mentioned earlier, the conditions of failure to regenerate are different in young larval limbs after denervation. Contact between wound epithelium and internal tissues is made, but no fibrous membrane develops under the wound epithelium. Instead, the wound epithelium continues to exert its histolytic action until the entire limb has disappeared. As in adult limbs, there is no growth of the products of dedifferentiation into a regenerate in the absence of nerves. However, a regenerate forms from these dedifferentiated tissues very rapidly when nerves re-enter the region.⁴⁷ A partial explanation of the difference in response of young and older animals is that older tissues have acquired a greater propensity for fiber formation.

These differences between young and old denervated limbs are paralleled by those exhibited in x-rayed limbs. Sufficient dosage to prevent regeneration of larval limbs is followed by dedifferentiation and resorption of entire limbs.³ Not so with older limbs; x-rayed and amputated limbs of large axolotls soon develop a scar beneath the epithelium and fail to regenerate. The argument that a fibrous scar acts as a barrier between epithelium and internal tissues, thereby preventing dedifferentiation, is supported by two observations from the irradiation experiments. X-rayed appendages of axolotls which had been amputated and had already formed a scar beneath the wound epithelium, were made to regenerate normally by removing the scar.¹⁸ The second observation is that young limbs x-rayed twelve days after amputation do develop a fibrous layer beneath the wound epithelium and do not dedifferentiate.⁴ In this case, a large blastema was already present and its cells were about to differentiate when the limb was x-rayed.

No theory of nerve action during regeneration can stand unless it can encompass two reports which are, at first, disconcerting, namely, that after transplantation of limbs nerves are not necessary for regeneration. It would seem unwise to doubt these reports, coming as they did, independently, from two of the best laboratories for the study of regeneration. If we accept them as true, and I think we must, our theory must fall unless transplantation itself causes the changes in a limb which we attribute to nerve action. It is fortunate that Polejaiev transplanted tadpole limbs of the same stage as Schotté and Harland denervated. Polejaiev reported regeneration in the transplanted limbs before nerves had an opportunity to re-enter them,²⁵ while Schotté and Harland, without transplanting, found no regeneration after denervation.⁴⁸

What does transplantation do to a limb? The answer is found in a

quotation from Polejaiev and Ginzburg³⁰ (see also¹⁷): "In a control at stage IIa subjected to simple limb amputation, the tissue differentiation is preserved in its essential features: the skeleton does not disintegrate, the muscle patterns maintain their integrity; mesodermal cells of the regeneration rudiment do not accumulate on the amputation wound surface under the epithelium. In case of limb autotransplantation, dedifferentiation of tissues takes place: cartilaginous epiphyses are transformed into dense mesenchymatous thickenings; muscle patterns disintegrate, forming a homogeneous mass of mesenchymatous cells which, assuming a round shape and moving towards the epithelium of the amputation wound surface, form the mesodermal part of the regenerating rudiment."

This is strong supporting evidence for the first part of the theory, namely, that nerves act by causing dedifferentiation (except in very young tissues).

Schneider has also reported regeneration without nerves after transplantation.⁴⁰ By transplanting a piece of axolotl limb to the region of the back, regeneration of poor feet was obtained. He also stated that regeneration was faster and better when the sciatic nerve was deviated along with the transplant. These facts are in no way incompatible with the second part of the theory, namely, that nerves also support regeneration by supporting growth without premature differentiation.

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Discussion of the Paper

DR. F. G. BUTLER (*Princeton University, Princeton, N. J.*):

One of the points I should like to discuss is whether, at the present time, we have final convincing evidence that regression does not occur in amputated nerveless limbs of adult urodeles. It was my understanding from listening to Dr. Rose that he has found no regression in the nerveless limbs in his experiments. On the other hand, I believe that in unpublished experiments Dr. Schotté has found that regression occurs in adult denervated limbs after amputation and prolonged absence of nerves. I should like to ask Dr. Rose how long he maintained the limbs in his experiments in a nerveless condition. It seems to me that, in this problem, time is a very important factor. It is not so much a question whether regression in an amputated limb takes place during a 30- or 60-day period of nervelessness, as whether it will take place during a 4- or 6-month period. Certainly, extreme regression takes place in larval limbs. Can it be that the situation is so different between the larva and the adult? In my opinion, we should have data from experiments on adults carried over a long period of time before we endeavor to answer this question positively.

It is my understanding from Dr. Rose's paper that he regards the epidermis as contributing to the blastema. It is possible that cells from the epidermis pass into the blastema, but do they form a functional component of the blastema? I ask this question not as a criticism of Dr. Rose's work, but rather for information. One of the most involved problems with which I am acquainted is that of endeavoring to recognize the source of cells which contribute to the regeneration blastema. Is there clear evidence that cells from the epidermis not only enter the blastema region, but also actually take part in regenerative activity?

Lastly, I should like to mention the matter of blastema age and its relation to limb regression. My work on regeneration has been primarily with urodele larvae and I am basing my statements chiefly on these.

I feel that the time has come when we can no longer simply refer to a regeneration "blastema." A newly formed blastema is a far different structure, morphologically and physiologically, than an older one. The blastema is a continually changing structure and at different times exhibits different types of activity. So far as regression is concerned, a young blastema is incapable of preventing a nerveless larval limb from undergoing regression; the presence of an older blastema, however, prevents a limb from regressing. I should like to suggest, therefore, that, in studying the physiology of regeneration, in adults as well as in larvae, the age of the blastema always be taken into account. It seems to me that this is an important consideration in the type of blastema which Dr. Rose is studying in his salted limbs.

DR. S. M. ROSE:

We have kept animals whose limbs had been denervated and amputated for as long as five months. Some of these animals began to regenerate limbs after several months' delay, others formed the type of callus shown in FIGURE 10, and still others formed practically no new tissue. There was no measurable regression in any of these animals except in one case, and in that only after the subclavian artery had been inadvertently severed. Judging with the work of Walter and of Singer as a basis, I think that these groups are different because of difference in nerve number. They are alike in failing to regress. Some other factor seems to be more important in causing regression than a nerve number below that necessary for limb regeneration. It would be very interesting to know whether adult limbs which had been maintained absolutely nerveless for a long time would, in time, begin to regress. As I remember, Dr. Schotté did believe that nervelessness for a long time would be followed by regression. Recently, in a discussion, he stated that regression had been obtained in adults after denervation if the amputation was performed through a regenerate. He did not speak of regression of older denervated tissues. I think that the question of the amount of regression after long periods of nervelessness is still unsettled and that we must await a projected paper by Dr. Schotté which will more fully cover the problem of delayed regression.

In our present work, it is clear that nerves play an important role in dedifferentiation. Without them, there is scarcely any dedifferentiation. This is very different from the situation in young larvae. I do believe that there are real differences between larva and adult; both the denervation and x-ray radiation experiments indicate it. My guess is that a difference originally quantitative, which we see reflected in the ability to produce collagen, is the basis for the resulting qualitative difference between young larva and adult. An initial study of some of the general age changes as they affect regeneration has been made by Miss Goodwin.¹⁰ Much more work on this important problem is needed.

I do not know whether epidermal cells enter the blastema and later

differentiate into "mesodermal" tissues. There is direct evidence that epidermal cells pass into the blastema. This has been described by Godlewski,⁸ by Hellmich,^{13a} by Neukomm,^{21a} and by Rose.³⁵ Godlewski, Neukomm and I were almost sure that we could see intergradations between epidermal cells and cells of the blastema. There is also the experiment by Umanski⁶³ indicating that a blastema may form from untreated skin transplanted to an x-rayed limb. That skin contained both epidermis and dermis, either or both of which can be suspected. We have unpublished studies from which it appears certain that vitally stained cells leave the epidermis and enter the blastema. Later, a few chondrocytes can be found containing the vital dye. Again, uncertainty creeps in and this cannot be considered evidence that epidermal cells become chondrocytes, because many of the epidermal cells are phagocytized and their stained granules transferred to their mesodermal predators. Hellmich, without using vital dyes, came to the conclusion that all of the epidermal cells in a blastema are destroyed. That may be true, but the observations above and other considerations³⁵ have kept alive the unproven idea that the epidermis may be one of the sources of blastema cells. A better test of the idea is needed than any used so far.

I fully agree with Dr. Butler that consideration of the age of a blastema is important. I should also like to say that knowledge of biochemical changes during this period would be very useful.

QUANTITATIVE STUDIES ON LOCOMOTOR RESPONSES IN *AMBLYSTOMA* LARVAE FOLLOWING SURGICAL ALTERATIONS IN THE NERVOUS SYSTEM

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Introduction

OVER an extended period of years, the author has been interested in the capacity of various portions of the embryonic nervous system of *Amblystoma* to undergo structural and functional readjustments under conditions by means of grafting.

One of the earliest experiments in this connection consisted in substituting trunk spinal segments (6, 7, and 8) from a donor embryo for the brachial segments (3, 4, and 5) of a host embryo. The embryos ranged in age from those with completely closed neural folds (stage 21) to those with a prominent tail bud (stage 30). A histological study of the cords in host larvae, approximately 50 days after the operation, showed that the grafted segments in the new position had undergone a volume increase and cellular hyperplasia which approximated the size and cellular content normally characterizing the brachial region of the cord. The forelimbs in 50 per cent of the cases exhibited normal function and were supplied by a typical brachial plexus, whose segmental contribution took origin from the grafted segments. These and other experiments dealing with the interchange of various spinal segments and the effects upon cellular proliferation, showed that the number of cells normally characterizing a given region is not inherently and irrevocably fixed. Some regions, however, are more plastic than others. Some have a greater "potential" for proliferation than others, regardless of their axial position.*

In some recent experiments (Detwiler, 1945) upon the embryonic brain, the presumptive hemispheres were excised in order to study any possible morphogenetic effects of their absence upon the development of the remainder of the brain, particularly the medulla. It had been shown by Burr (1916a) that the cerebral hemispheres of *Amblystoma* are dependent upon the intactness of the nasal sacs for their complete development. He also showed (1916b) that, when a cerebral hemisphere

* For a discussion of the morphogenetic responses of various spinal segments in altered positions see Detwiler, 1936. Hamburger (1946) has studied the proliferation of cells in mechanically isolated portions of the chick spinal cord, and has discussed the results in relation to earlier findings on the amphibian cord following certain experimental procedures.

is removed from embryos,* it will regenerate only if the nasal placode is left intact. The stimulus for regeneration was assigned to the ingrowth of the olfactory nerve fibers, a contention which received support by later experiments (1930).

My own experiments upon the forebrain corroborated Burr's results in that there was no evidence of regeneration of the hemispheres. In these experiments, which were conducted on Harrison's stage 21±,† the excised region included the presumptive nasal placodes and the eye rudiments. Both unilateral and bilateral excisions were made. Since it was doubtful whether embryos lacking the entire forebrain, eyes, and nasal placodes could lead an independent existence beyond the yolk resorption stage, many were fused parabiotically with normal embryos to serve as nurses and at the same time as controls for the operated components. Others were allowed to develop as "free" individuals.

External malformations in the shape of the head became visible fairly early. The growth of the upper jaw was greatly reduced, resulting in a marked protrusion of the lower jaw (FIGURES 1 and 2). In cases of unilat-

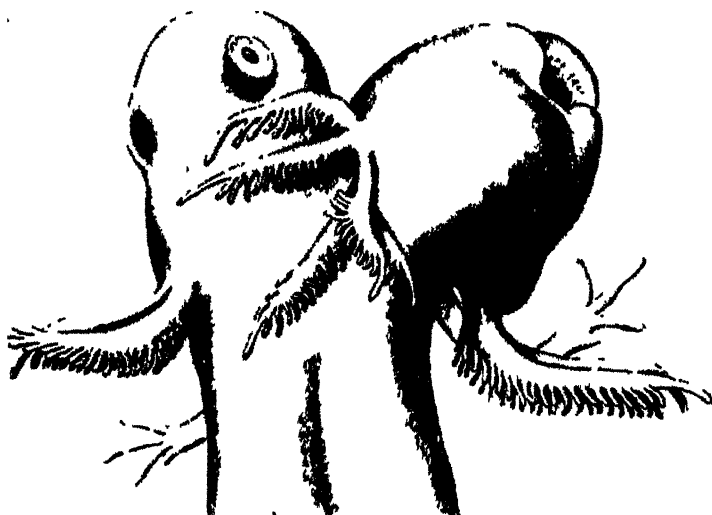


FIGURE 1. Parabiotic twin (FBEBP20) 26 days after operation. x8. The right component lacks both cerebral hemispheres, eyes, and nasal organs.

eral excision, this condition existed only on the side of operation (FIGURE 3).

When the larvae had reached the feeding stage, most of the non-parabiosed individuals, despite the absence of forebrain, eyes, and nasal placode, exhibited snapping reactions when a needle was moved gently

* Stages of operation not indicated.

† At stage 21 the neural folds are completely closed.

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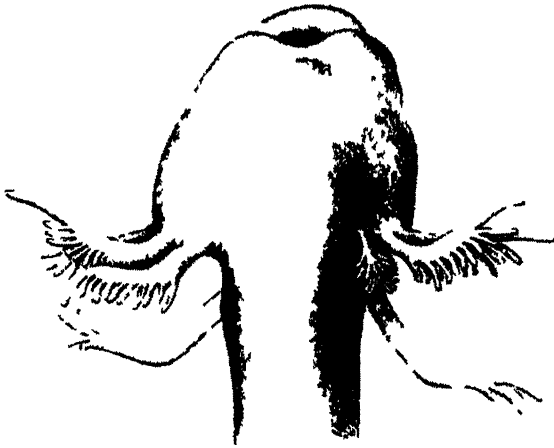


FIGURE 2 *Amblystoma* larva (FBEB44) 26 days after operation \8. The fore-brain, eyes, and nasal placodes were removed from the embryos in stage 21.



FIGURE 3 *Amblystoma* larva (FBE21) with absence of the right hemisphere, the ipsilateral eye, and nasal sac, 30 days after operation \8.

back and forth along the side of the head. They were capable also of

feeding upon daphnia and small enchytraeid worms. Sharrer (1932) had indicated previously the significance of the lateral line sense organs in the characteristic snapping reactions. It had also been shown later (Detwiler and Copenhaver, 1940) that the growth rate of larvae lacking eyes and nasal organs may keep pace with that of controls under conditions of maximal feeding. This indicated clearly that the lateral line sense organs alone may constitute an adequate receptor apparatus for the detection of food in motion.

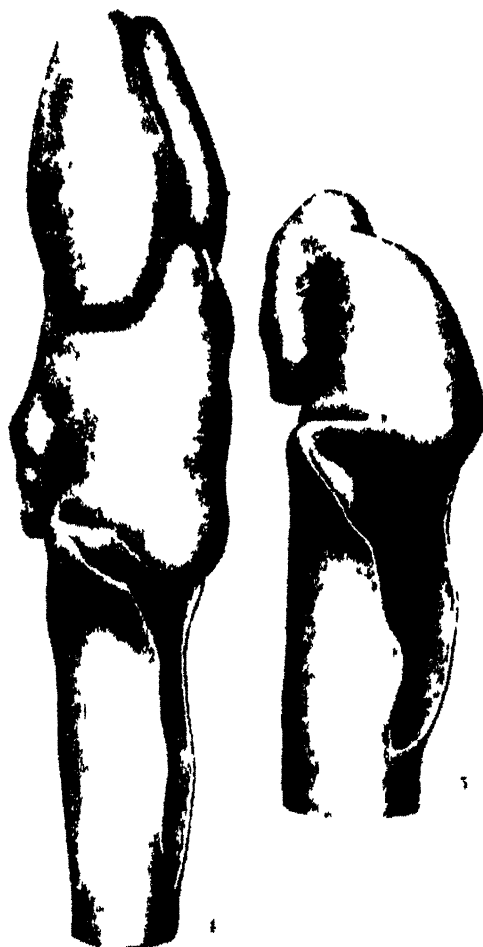


FIGURE 4 Wax reconstruction of the brain (dorso-lateral view) of the normal component of parabiotic twin FBEB22 $\times 20$

FIGURE 5 Wax reconstruction of the brain (dorso-lateral view) of the operated component of parabiotic twin FBEB22 $\times 20$ Note lack of hemispheres and the dorsal portion of the diencephalon

The intake of food, and the growth of the larvae lacking the forebrain, eyes, and nasal placodes, was markedly curtailed as compared with the controls. Spontaneous behavior in general was greatly reduced, both quantitatively and qualitatively, especially the foraging reactions, regardless of the amount of food available. The mean length of the larvae at 45 days of age was slightly more than 50 per cent of the length of maximally fed normal animals of similar age. Nevertheless, the motor activities concerned with lurching, engulfing food, chewing, and swallowing were carried out in an integrated manner, although they were decidedly less vigorous than in larvae with intact hemispheres. In the parabiotic twins, the eating function was taken over largely by the more active conjoined normal component, but here, too, the operated component was seen to snap and engulf food. Although actual measurements were not recorded for all cases, it may be said that the growth of the twins was greater than that of the operated "free" individuals, but not as great as in the normal control larvae.

Wax reconstructions of the brains of the 2 components in twin 22 are shown in FIGURES 4 and 5. The operated component not only lacks the hemispheres, but the dorsal portion of the diencephalon is also wanting. The hypothalamic region and the pituitary gland (both glandular and neural portions) are present and essentially normal. This situation was characteristic of all cases whether the individual was parabiosed or not. The defective diencephalon is interpreted as resulting from partial ablation during the operation rather than to any morphogenetic influence due to the absence of the hemispheres.

Despite the complete absence of the hemispheres, an incomplete diencephalon, and a possibly smaller mesencephalon, the medulla in all cases showed no significant reduction in size. The medullas of the operated components were somewhat shorter than those of the normal components of the twins (FIGURE 6, *cf.* A and B), but their volume in all cases studied, except one, ranged from 92 to 99 per cent of normal (Detwiler, 1945, Table 1).

In cases with unilateral excision of the forebrain, there occurred a compensatory enlargement of the contralateral nasal sac and the adjacent hemisphere. The single hemispheres in 4 cases studied showed volume increase ranging from 73 to 88 per cent of the volume of both hemispheres in a control larva of similar length (Detwiler, *op. cit.*, Table 2). A histological study of nuclear size forced the conclusion that the compensatory enlargement of the hemisphere was accomplished by a cellular hyperplasia.

The capacity of the young larvae lacking hemispheres, eyes, and nasal organ to obtain food and to lead an autonomous existence, led to experiments designed to test the behavior of larvae lacking the midbrain.

Nicholas (1930) excised the embryonic mesencephalon and replaced it with a graft consisting of the forelimb and adjacent pronephric rudiments, thus isolating the forebrain from the remainder of the nervous

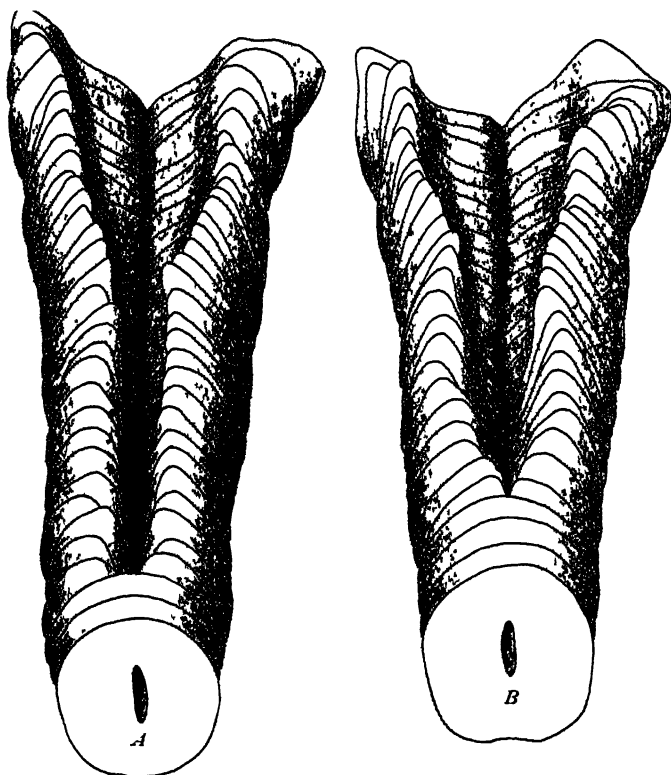


FIGURE 6. Graphic reconstruction of the medullas of normal component (A) and operated component (B) of parabiotic twin FBEB3. The operated component lacks the cerebral hemispheres, eyes and nasal sacs. $\times 40$. (See FIGURE 1.)

system. He found that the early responses (*C* and *S* reactions) were carried out normally. He said little about the effects of the operation upon behavior of older larvae, except that spontaneous movements were not present and that vigorous swimming movements could be elicited by stimulation. These apparently diminished in amount as the animals became older. Nicholas did observe that his larvae were unable to obtain food, and this failure was regarded by him as due to the fact that "the removal of the mesencephalon created a block in the nervous system so that normal feeding responses could not be obtained."*

Experimental Results

In my own experiments upon the mesencephalon, three types were performed: (A) excision of the mesencephalon from embryos in stage 20-21 and replacement by the first and second spinal cord segments

* In cases where the larvae were kept beyond the feeding stage (stage 46), the animals were fed by artificial means.

from a donor embryo in stage 22-23; (B) end-to-end reversal of the entire midbrain in embryos of stage 22; and (C) unilateral reversal of the midbrain in embryos in stage 20-21, involving reversal of the antero-posterior axis only.

Excision of the Mesencephalon and Its Replacement by Cord Segments I and II. This procedure differs from that of Nicholas (1930) in that nervous continuity is maintained between the fore- and hindbrain. The majority of the larvae at the feeding stage were unable to eat. Several exhibited feeble snapping reactions but were unable to obtain their prey. Only 2 cases in 24 were recorded as having eaten, and their intake was very meager. In most cases, the jaws were immobile and held slightly apart. This condition readily explains the inability to capture *Daphnia* in those cases where snapping was attempted. The incidence of kyphosis was very low; there were only 2 cases in this series which developed this condition. This is in marked contrast with the results obtained by Nicholas, all of whose larvae developed a marked ventral bending of the head and tail. The fact that the larvae in the present experiments developed as straight individuals naturally favored a critical study of their swimming responses.

Observations upon Swimming Responses. All the early larvae exhibited normal C and S flexures and swimming responses. In later stages, however, despite previous manifestations of an adequate intraspinal swimming mechanism, the larvae showed progressive failure both in their responses to tactile stimulation and in their locomotor ability. The method of studying behavior was as follows: Individual larvae (normal and mesencephalonectomized of similar stages) were placed in a Syracuse dish and stimulated 25 times at approximately 5-second intervals. This was done by stroking the skin over the myotomes with a human hair, according to the method of Coghill (1909). At the end of 25 stimulations, the number of "misses" was recorded. In addition to studying the responses to tactile stimulation, the distance traveled in response to 25 stimulations was measured. This was done as follows: A circle, the diameter of which equaled the inside diameter of a Syracuse dish, was drawn upon a card. This was divided into 10 sectors of arc (36° each). The sectors were numbered successively from 1 to 10 both clockwise and counterclockwise, and the card was then placed beneath the dish so that the circle coincided with the inner wall of the dish. A larva was placed adjacent to the inner wall of the dish and headed in a clockwise direction. It was then stimulated and the number of sectors traveled before coming to rest recorded. This was done for 25 stimulations at approximately 5-second intervals. At the end of these, the number of positive reactions and the total distance traveled in units (sectors of arc of 36° each) were recorded for each larva. Young larvae are strongly thigmotactic and nearly always swim along the wall of the dish. In order to avoid occasional short-cutting, the device was improved by placing

a glass ring within the Syracuse dish which provided a "moat" about 7 mm. in diameter. The improved device is shown in FIGURE 7.

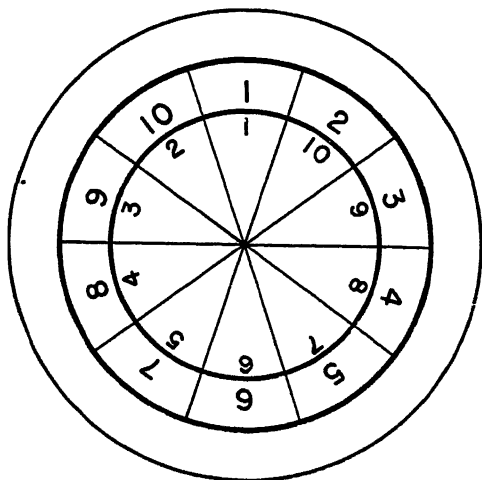


FIGURE 7. An improved device placed beneath a Syracuse dish for quantitating the distance traveled by young *Amblystoma* larvae (stages 39 to 46 and beyond). The outer heavy circle corresponds to the inner wall of the dish; the inner heavy circle indicates a glass ring the height of the dish. The space between the two represents a "moat" approximately 7 mm. in diameter. Each larva was placed in the moat and stimulated 25 successive times at approximately 5-second intervals, and the total distance traveled was recorded in units (sectors of arc). The glass ring has been added to the original device (Detwiler, 1945, Figure 1) to prevent larvae from occasionally short-cutting as they swim along the wall of the dish (n. text).

Records were made upon 2 separate groups each consisting of 4 control and 6 experimental larvae. The data on these 2 groups are shown in FIGURES 8, 9, 10 and 11. FIGURES 8 and 10 show the responses to tactile stimulation, while FIGURES 9 and 11 record the average distance traveled by the larvae at the stages indicated. It is seen that, beyond stage 41, the number of "misses" among the experimental animals was indeed very striking. Although there was some variation in the average distance traveled by the normals at the various stages (solid columns), it is clear that the propulsive power of the experimental larvae was greatly reduced (stippled columns). In no single experimental larva from stage 41 on did the maximum swimming response equal the minimum response of any of the normal larvae.

It is apparent from the findings that the execution of normal locomotor responses quite early in larval life depends upon the intactness of the mesencephalon. Up to about stage 40, the absence of this structure appears to have little or no effect upon the swimming activities, which are autonomous in the sense that they apparently can be carried out entirely independent of any structural connection with the mid-brain. The rapid falling-off in the locomotor capacity at this period (stage 40-41) is regarded as evidence of the importance of the midbrain tectum

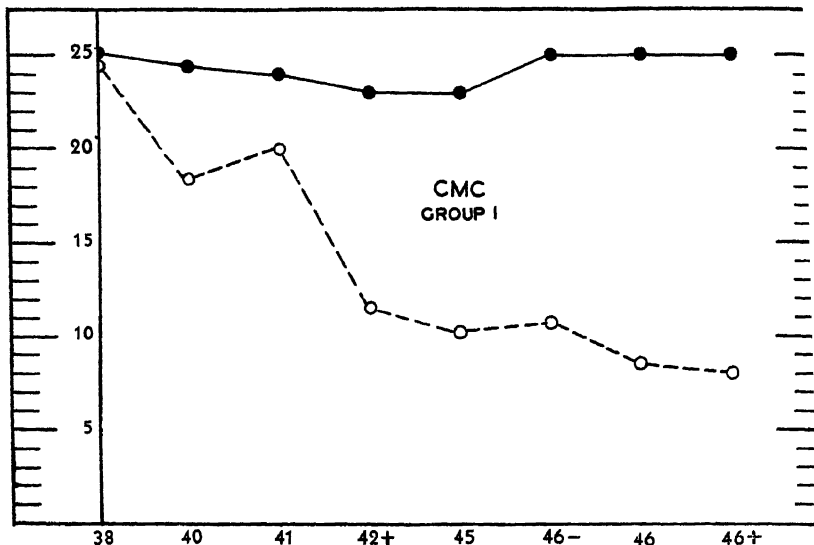


FIGURE 8. Graph showing locomotor responses of *Amblystoma* larvae (CMC-group 1) to 25 successive tactile stimulations at 5-second intervals. Continuous line shows the average for 4 normal control larvae; broken line shows average for 6 larvae in which the midbrain was replaced by the first two segments of the spinal cord. X axis represents stage; Y axis indicates number of responses.

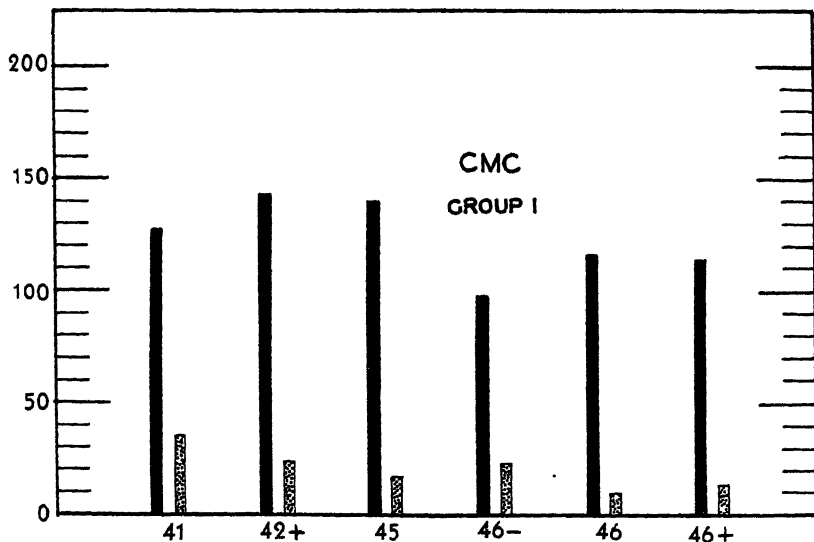


FIGURE 9. Graph showing total distance traveled by the larvae in group 1, in response to 25 successive stimulations at 5-second intervals. The solid columns show the average distance covered by 4 control larvae; the stippled columns show the average distance traveled by 6 larvae whose midbrain was replaced by the first and second spinal cord segments. X axis designates stages; Y axis represents distance in units (sectors of arc as shown in FIGURE 7).

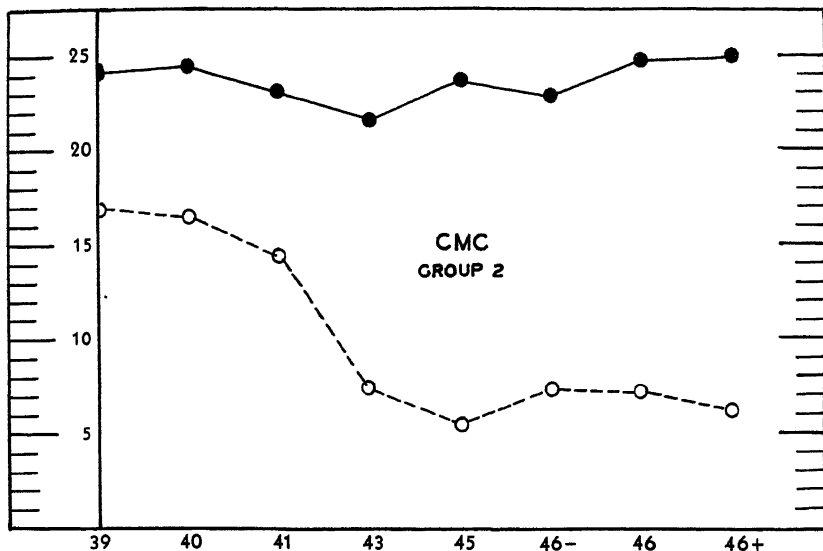


FIGURE 10. Graph showing locomotor responses of the larvae in group 2 (CMC) to 25 successive tactile stimulations at 5-second intervals. Continuous line shows average for 4 control larvae; broken line shows average for 6 larvae with midbrain replaced by spinal cord segments 1 and 2. X axis indicates stages; Y axis indicates number of responses.

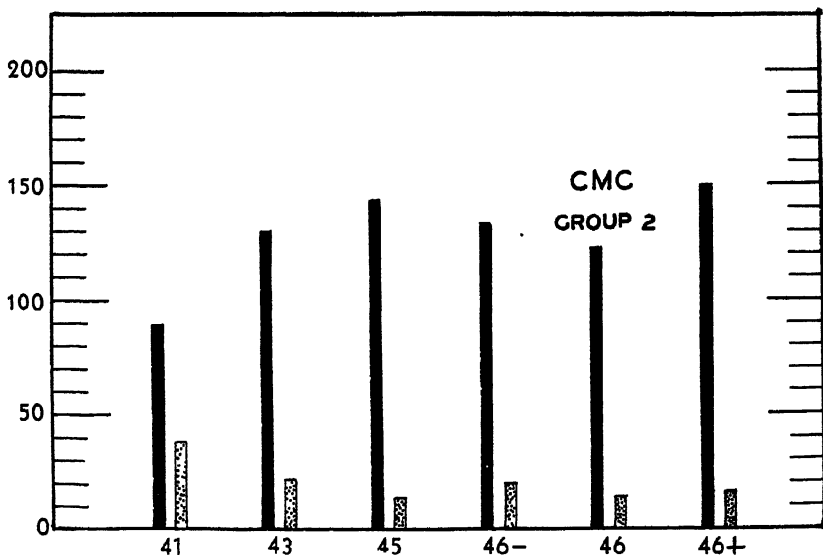


FIGURE 11. Graph showing total distance traveled by the larvae in group 2 (see explanation of FIGURE 9). X axis designates stages; Y axis represents distance in units (sectors of arc as shown in FIGURE 7).

in coordinating and sustaining these functions. Crossed and uncrossed tracts arising in the tectum invade the medulla as the tecto-bulbar tracts. These make synaptic connections with tracts which arise in the medulla and invade the cord as the bulbo-spinal tracts (Herrick, 1914). However, Herrick (1939) says that some of the fibers arising in the tectum probably invade the spinal cord directly as the tecto-spinal fibers. According to him, the uncrossed tecto-bulbar tracts develop earlier than the crossed fibers of this system. These latter appear in preparations "subsequent to early swimming stages." In the earlier feeding stages, he finds that essentially adult relations have been attained.

It should be pointed out here that larvae lacking the midbrain are still capable of some locomotor activity, *i.e.*, the intra-spinal mechanism can still function, but to a greatly diminished degree (FIGURES 9 and 11). The evidence seems clear, however, that the spinal apparatus, which is entirely adequate for normal propulsion in the early stages ($37-40\pm$), later falls under mesencephalic control.

Although no attempt was made to trace out the pre-otic cranial nerves, it would appear that the failure of the animals in this series to eat (*vide supra*) was largely due to the defective development and displacement of the V-VII ganglionic complex as a result of the operation.

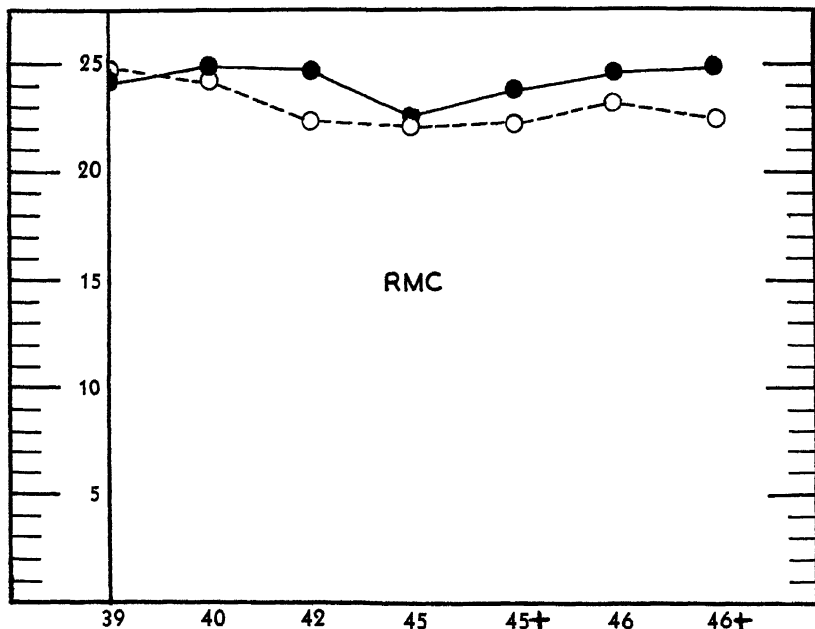


FIGURE 12. Graph showing locomotor responses to 25 successive tactile stimulations at 5-second intervals. Continuous line shows average for 4 normal control larvae; broken line shows average for 6 larvae in which the midbrain was reversed end for end at stage 22. X axis designates stages; Y axis indicates number of responses.

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Failure to cut precisely between embryonic mesencephalon and medulla resulted in the inclusion of the anterior end of the medulla in many excisions—thus involving either the inclusion of or the disturbances to the adjacent V-VII complex.

End-to-End Reversal of the Mesencephalon. As in the series described above, the early *C* and *S* responses were normal. The responses of larvae to tactile stimulation and their motor ability were tested in the manner described above. The results are shown in FIGURES 12 and 13,

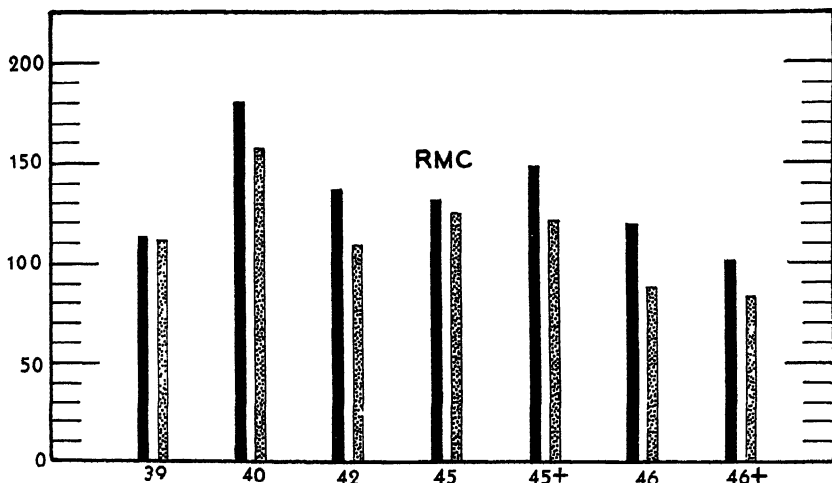


FIGURE 13. Graph showing total distance traveled by larvae in response to 25 successive stimulations at 5-second intervals. The solid columns show the average scores for 4 control larvae; the stippled columns show the average scores of 6 larvae in which the midbrain was reversed end for end in stage 22. X axis designates stages; Y axis indicates distance in units (sectors of arc as shown in FIGURE 7).

and they reveal the fact that, despite complete reversal of the midbrain, the larvae responded to tactile stimulation nearly as well as did the controls. More striking, perhaps, is the fact that the motor capacity was only slightly lowered. This is in marked contrast with the greatly curtailed motor capacity of those lacking the midbrain (*cf.* FIGURES 9 and 11). A microscopic examination of the larvae in this series showed that, in 4 of the 9 cases studied, there was no detectable evidence that the midbrain had been reversed (FIGURES 14 and 15). In 5 others, which were incapable of feeding, there were morphological deficiencies which could well account for this defect (Detwiler, 1946a, p. 129).

Unilateral Reversal of the Midbrain. In these experiments, the right half of the mesencephalon was excised and replaced by the left half from a donor embryo, thus reversing only the antero-posterior axis. The young larvae, like those of the two previous series, showed normal developmental behavior. No quantitative records were made of the motor activities until stage 46, when 8 larvae were tested along with 6 normal

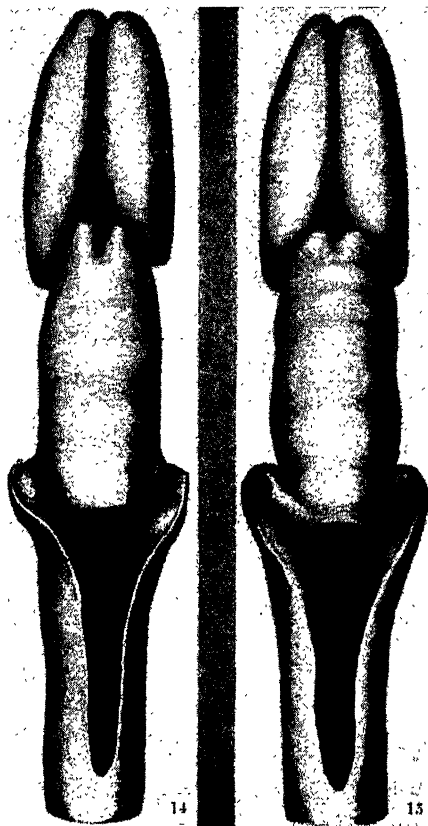


FIGURE 14. Reconstruction model of the brain of a normal larva (48 days, 35 mm.) for comparison with the brain of case RMC60 (with reversed mesencephalon) as shown in FIGURE 15. x40.

FIGURE 15. Reconstruction model of the brain of case RMC60 (42 days, 38 mm.). The embryonic midbrain was reversed end for end in stage 22 (*cf.* FIGURE 14). x40.

controls of the same developmental stage. The results showed that the average distance covered by the experimental animals equaled that of the controls. Certain developmental abnormalities were encountered in some of the cases in this series; these have been described previously (Detwiler, 1945, p. 132). One of the most interesting conditions consisted in the failure of the graft to fuse with the cephalic and caudal stump of the host brain. In consequence, the graft developed into an isolated, bilaterally symmetrical whole, and the excised midbrain half was restored by regeneration from the contralateral intact half (Detwiler, 1946b). These cases are of especial interest in showing that, at this stage of development, half a mesencephalon is capable of restoring the whole. The same capacity had been shown previously for the medulla (Detwiler, 1944).

Locomotor Responses of Larvae Lacking the Cerebral Hemispheres. Although the general behavior of larvae lacking the hemispheres had been observed prior to those without the midbrain, no quantitative studies on locomotor responses were made at the time. Consequently, the experiments were repeated and the motor ability tested with the device shown in FIGURE 7. Two groups each consisting of 5 operated and 5 con-

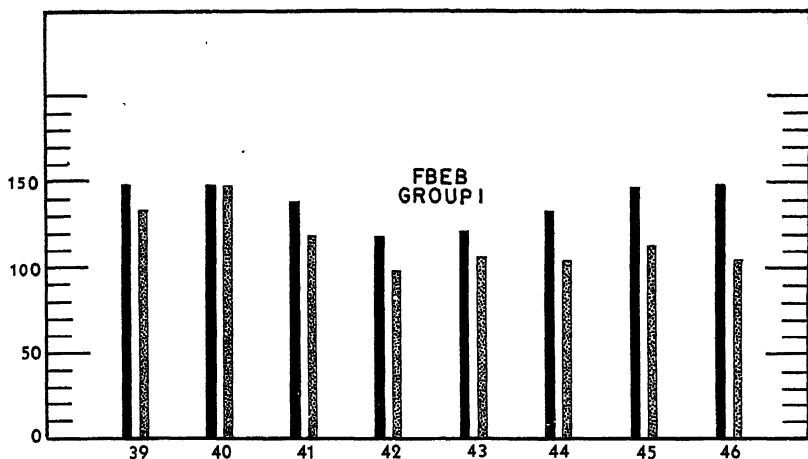


FIGURE 16. Graph showing total distance traveled by the larvae in group 1, in response to 25 successive stimulations at 5-second intervals. The solid columns show the average scores for 5 control larvae; the stippled columns give the average scores for 5 larvae lacking the cerebral hemispheres and the dorsal portion of the diencephalon. X axis designates stages; Y axis represents distance in units (sectors of arc as shown in FIGURE 7).

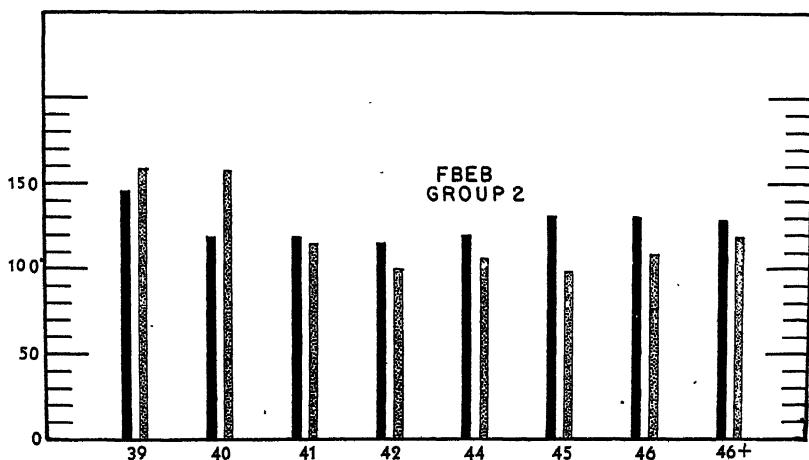


FIGURE 17. Graph showing average scores of 5 control larvae (solid columns) and 5 experimental larvae (stippled columns) in group 2. See explanation of FIGURE 16.

trols were tested from stages 39 to 46+ (feeding stage). The averages of the scores for both normal and operated larvae in the two separate groups are illustrated graphically in FIGURES 16 and 17. These show that the operated larvae in the earlier stages do somewhat better, when compared with their controls, than they do in later stages (stages 45 and 46). The lowered motor ability, however, is only slight as compared with the extreme motor incapacity of larvae lacking the midbrain. The lack of the forebrain also had no effect upon the responses to tactile stimulation. The number of "misses" to trunk tactile stimulation among the operated larvae was no greater than among the controls. This is in marked contrast with the failure exhibited by the mesencephalonecto-

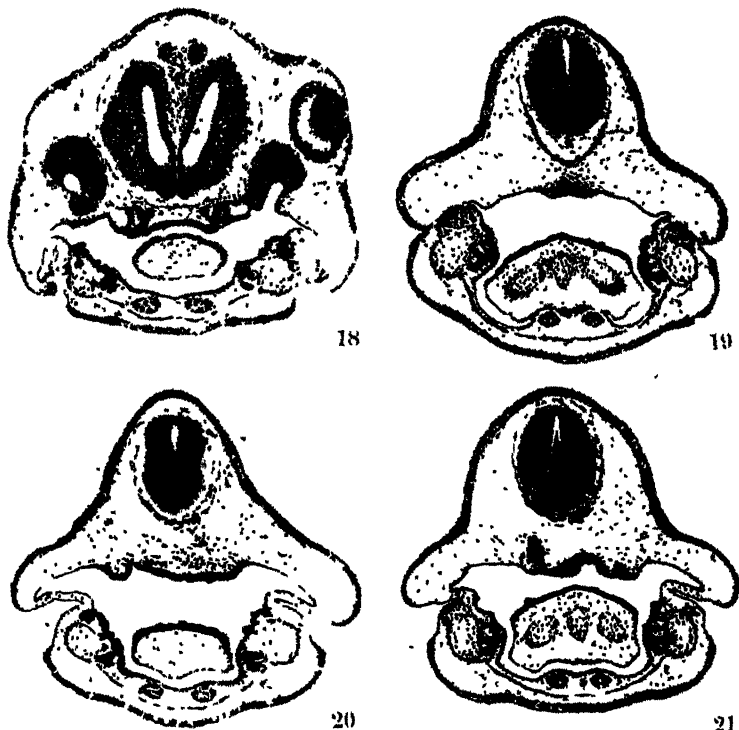


FIGURE 18. Photomicrograph of transverse section (2-1-4) of a normal larva (CA) at stage 46 (feeding stage) showing cerebral hemispheres, nasal placodes, and an eye. It is the thirty-first section caudal to the first appearance of the brain in section. $\times 33.3$.

FIGURE 19. Photomicrograph of section (1-4-1) from case FBEB (4A) at stage 46. It is the seventh section caudal to the first appearance of the brain in section. The larva is completely devoid of cerebral hemispheres and the dorsal portion of the diencephalon. $\times 33.3$.

FIGURE 20. Photomicrograph of section (1-3-4) from case FBEB (25A). It is the seventh section caudal to the first appearance of the brain in section. The larva lacked the hemispheres and the dorsal portion of the diencephalon. $\times 33.3$.

FIGURE 21. Photomicrograph of section (1-4-2) from case FBEB (25). Conditions same as those described for FIGURES 19 and 20. $\times 33$.

mized larvae, which showed a striking decline in response to tactile stimulation subsequent to stages 40 and 41.

Morphological Observations. Ten operated larvae and 1 control subsequent to stage 46 were fixed and studied in serial transverse sections. All 10 cases showed a total lack of the hemispheres. In addition, the dorsal portion of the diencephalon was also wanting. The hypothalamic region and pituitary gland were present in most cases, but in several this region was defective.

Four of the sectioned animals are illustrated in FIGURES 18–21. FIGURE 18 is a photomicrograph of the thirty-first section caudal to the beginning of the hemispheres in a control larva (1). The hemispheres are prominent, as are also the nasal placodes and an eye. The microphotographs shown in FIGURES 19, 20, and 21 represent the seventh section caudal to the first appearance of brain tissue in the operated animals. The brain in these sections is readily recognizable as mesencephalon.

Many of the animals of this series failed to feed. Fight larvae which did eat followed a behavior pattern similar to those described previously (Detwiler, 1945), viz., curtailed spontaneous behavior, rather feeble lurching, reduced food intake, and marked reduction in growth. The activities involved in lurching, engulfing, chewing, and swimming were performed in an integrated manner.

Motor Responses Following the Excision of the Right Mauthner's Neuron and of the Right Ear Vesicle. In teleost fishes and in larval Amphibia, there exist 2 giant neurones known as Mauthner's fibers. These have been of considerable interest to neurohistologists because of certain anatomical features they exhibit—mainly the gigantic size of the cell, the high degree of differentiation, the characteristic position in the medulla, the extensive dendritic connections, the internal cell structure, and the nature of the synapses.

Studies by different authors upon the anatomical connection of these cells in various forms have resulted in several suggestions regarding their function.

In *Amblystoma*, the two perikarya occupy a lateral position in the medulla at the level of the entrance of the VIII nerve (FIGURE 22), and the axones, after decussating in the medulla, course caudally through the spinal cord in a ventral position (FIGURE 23) where connections are made with both motor and intercalary cells (Coghill, 1934).

With regard to the function of this cell, Bartelmez (1915) says: "The reduction of the latent period by elimination of the synapses and the highly medullated character of the system have led me to believe that we are dealing here with a reflex in which speed and precision are very important, and I would suggest that it is this reflex which enables the animal to keep perfect control of its equilibrium in the most rapid and intricate movements." He also suggests that the great caliber of the axones, the broad contact surfaces in the synapse of the lateral dendrite,



FIGURE 22. Photomicrograph of section (1-3-5) through the medulla of case MPEB (2A) (39 days), showing Mauthner's cells, and their positional relation to the VIII nerve. $\times 50$.

FIGURE 23. Photomicrograph of section through the spinal cord at the level of the third spinal nerve showing position of Mauthner's fibers. $\times 66$.

and the highly insulated character of the whole path, all point to a very rapid reflex. The fact that Mauthner's cell, in addition to its connections with the VIII nerve roots, has connections with every center in the brain which receives impulses that may be used in equilibrium, strongly suggested to Bartelmez an important role in equilibratory reflexes.

Although much attention had been paid to the dendrite connections of the cell body in the brain, it was not until the observations of Coghill (*op. cit.*) that important and hitherto unknown relations of the axonal collaterals in the cord became known. Based on his studies, Mauthner's fibers are regarded as constituting an inhibitory system. Following his observations on the synaptic connections within the cord, Coghill says: "Upon the hypothesis that the action is inhibitory, a volley of impulses passing cephalo-caudal along Mauthner's fibers would inhibit local sensory-motor responses, represented by the cells of intercalated type, in favor of the total action pattern and at the same time would inhibit the antagonist of one side in favor of the agonist of the other, as represented by the cells of motor type. Such inhibition would facilitate the cephalocaudal progressing flexures which effect swimming. Possible interference of local patterns of action (reflexes) with the total action pattern of swimming would thus be prevented, and interference of the axial musculature of the right side with that of the left and vice versa, would be blocked."

Coghill's interpretations are in harmony with my own findings based upon the excision of these cells (Detwiler, 1927, 1933), where it was shown that the swimming reflexes of larvae lacking one or both neurones are inferior to those of normal animals. Larvae devoid of one Mauthner's neurone were found to exhaust more quickly than normals following repeated tactile stimulations. The swimming frequently was jerky and uncoordinated. There was no evidence from these experiments that the

lack of one of the Mauthner's neurones had any effect upon equilibration.

In excising a Mauthner's cell, the ear vesicle first had to be removed and subsequently replaced by a vesicle from a donor embryo. Histological studies showed that, when larvae lacking the Mauthner's cell exhibited equilibratory disturbances, they were referable to an abnormal ear or to defectivenervous connections, or both. It became apparent, from the results, that Mauthner's neurones are not indispensable to normal equilibrium. On the other hand, the absence of one ear was followed by marked equilibratory disturbances.

According to Coghill's view, the removal of Mauthner's fiber would release a normally present inhibitory effect on the local patterns of action and the interference between this and the total action pattern would be increased, as well as that between the axial musculature of both sides of the body. Regardless of the exact way in which the mechanism works, both the histological and physiological findings agree that this system is important in sustaining coordinated motor activity.

When the earlier experiments were made upon Mauthner's cells, no device was available for measuring the motor responses. It seemed desirable, therefore, to repeat the experiments and test their locomotor responses with the device shown in FIGURE 7.

The technique employed in removing the Mauthner cell consisted in first excising the ear vesicle. A small mass of cells was then cut away from the lateral wall of the medulla just medial to the ear region. This was followed by replacement of an ear vesicle from a donor embryo, using care that it was implanted with normal orientation. The operation was made on embryos in the early tail-bud stage (stages 27 and 28). For studies on comparative behavior, the ear vesicle alone was removed from a group of similar aged embryos, leaving the Mauthner neurone intact. In both groups, the *C* and *S* flexures and the early swimming responses were carried out in normal fashion. Beginning with stage 38, the motor responses were measured upon three groups as follows: (1) 10 normal control larvae; (2) 20 larvae from which the right Mauthner's cell had presumably been removed; and (3) 10 larvae lacking the right ear vesicle. This was done with the device shown in FIGURE 7. Each animal was stimulated 25 times, at approximately 5-second intervals, by stroking the trunk skin with a human hair as described above. The total number of units (sectors of arc) traveled by each animal following 25 stimulations was then computed, the average determined for the individuals in each group, and the standard error computed.* This procedure was carried out for each successive stage up to the feeding stage (44+) and then upon larvae 2, 4, and 7 days respectively after feeding.†

* I am indebted to Professor Herbert Elftman for subjecting my data to a statistical analysis and computing the standard error.

† In *A. punctatum*, the larvae begin to feed at stage 46. In these experiments, *A. jeffersonianum* was used and they were observed to begin feeding at a developmental period comparable in many respects to stage 44 of *A. punctatum*.

The data, based upon approximately 10,000 individual recordings, are given in FIGURE 24. An examination of this figure reveals a number of

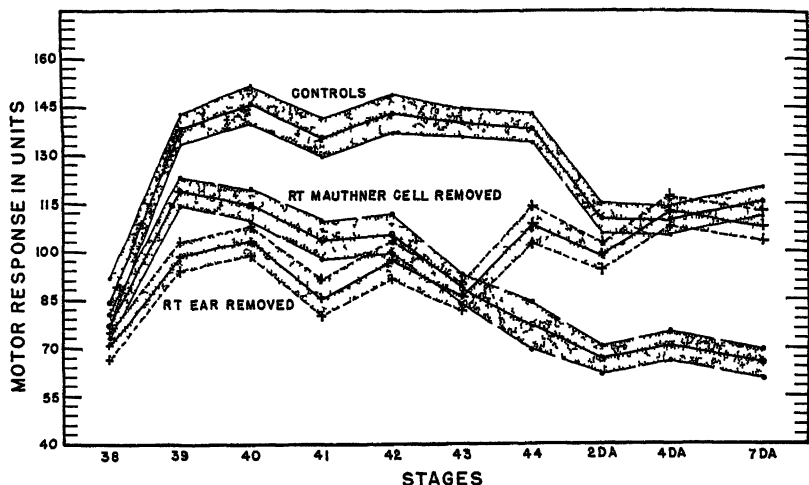


FIGURE 24 Graph showing average total distance traveled by larvae of *A. jeffersonianum* in response to 25 successive stimulations at 5-second intervals under the conditions indicated. The heavy central line for each curve represents the average, the line above and below represents the standard error. The curves are based upon the data obtained from 10 normal larvae, 20 with the right Mauthner's cell removed, and 10 with the right ear removed. Upon later histological examination, it was found that in 7 of the Mauthner neurone series the cell had not been excised (v text for explanation).

interesting facts. The larvae of all three groups made relatively low scores at stage 38, those lacking the ear being the lowest. At stage 39, there was a rise in the scores of all groups, but the controls markedly outdistanced those of the other two groups. Here, again, those lacking the ear made the poorest records; those lacking Mauthner's neurone occupied an intermediate position. From stage 40 on, there was a general overall decline in the motor ability of the group with theoretical absence of the Mauthner's neurone. Although there existed considerable variation at different stages, those lacking the ear showed marked improvement in their scores after stage 44, and at 4 days after the feeding stage their scores equaled those of the controls. Despite the improvement in their locomotor ability, these larvae lacking the ear still exhibited the characteristic equilibratory disturbances during locomotion. The behavior of larvae lacking an ear vesicle has been described by Greene and Laurens (1923) and subsequently by the author (1927, 1933). Such animals exhibit torsion towards the earless side. There is flexion of the arm on that side, with extension of the opposite arm. In swimming, the larvae rotate frequently on the longitudinal axis so as to exhibit a "corkscrew" movement. They may come to rest upside down, but more frequently land on the earless side. Spontaneous movements are usually less marked.

The larvae with theoretical absence of Mauthner's neurone showed no signs of improvement in their scores up to 7 days *post* feeding. Incomplete records of some older larvae of this group showed improvement in locomotor response, but the data were too scattered to be treated statistically. Viewing the curve as a whole, it is apparent that larvae lacking Mauthner's cell do not have the locomotor capacity of normal larvae. The fall in locomotor ability of this group began at stage 39. It may be pertinent to point out from microscopic studies of normal larvae that, at stage 39, Mauthner's cell shows some differentiation, which appears to be complete by stage 41.* According to the records (FIGURE 24), it is at this period when the motor response of this group began to decline (*cf.* controls).

In order to obtain evidence as to whether the Mauthner neurone had actually been excised, the larvae of this group were studied in serial transverse section. Whereas the group was originally made up of 20 larvae, only 16 were available at the end of the experiment. Both Mauthner's neurones were found to be present in 7 of the 16 animals. Most of these individuals, from which the right neurone had not been excised, also had low scores, but the average, on the whole, was higher than that of the group lacking this neurone. The low scores of the group with this neurone intact, as compared with those of the controls, leads one to suspect that, whereas the cell had not been excised, the operation may have disturbed the relations sufficiently to prevent normal connections of the cell body, resulting thereby in a lack of normal function. This theoretical explanation received some support from the fact that, in several cases, the perikaryon was displaced and in 2 cases was very small.

This series of experiments is regarded as incomplete, and it is planned to repeat the experiment and test the locomotor responses of much older larvae along with those of normal controls of similar age. In so far as they go, the results bear out former conclusions that the locomotor ability of larvae lacking the Mauthner fiber is inferior to that of normal animals.

Summary and Conclusions

1. With a device described in the text (FIGURE 7), the locomotor responses of young *Amblystoma* larvae have been quantified under the following conditions:

- (a) Control larvae for each group listed below.
- (b) Larvae lacking the cerebral hemispheres and the dorsal portion of the diencephalon.
- (c) Larvae with the midbrain replaced by the anterior end of the spinal cord.
- (d) Larvae with end-to-end reversal of the mesencephalon.
- (e) Larvae with unilateral reversal of the mesencephalon (reversal of A-P axis only).

* Based upon a microscopic study of Harrison's normal stages.

(f) Larvae lacking the right Mauthner's neurone, but with intact ears.

(g) Larvae lacking the right ear.

B. The data obtained from this group, where locomotor responses were measured from stages 39 to 46+, show that during these stages the motor capacity is only slightly lowered (FIGURES 16 and 17). The relative unimportance of the hemispheres in the general motor activities is clearly indicated.

C. Larvae without the midbrain, but with nervous continuity between fore- and hindbrain exhibit normal locomotor responses up to approximately stage 40. Thereafter, the motor capacity is greatly lowered (FIGURES 9 and 11). The data indicate that the swimming mechanism in the early stages is essentially spinal and autonomous, but that at approximately stages 40-41 it becomes subservient to mesencephalic control, probably concomitant with the development of the tecto-bulbar and tecto-spinal tracts.

D. Reversal of the mesencephalon only slightly lowers the locomotor capacity. The scores of larvae ranging from stages 39 to 46+ are in general of a magnitude similar to those exhibited by larvae lacking the hemispheres (cf. FIGURES 13 and 16).

E. Unilateral reversal of the midbrain (A-P axis only) has no effect upon the motor ability of the animal.

F. Animals lacking one Mauthner's neurone show significantly lowered scores as compared with those of control larvae (FIGURE 24). They differ from those lacking an ear in that the scores did not improve in the later stages under investigation.

G. The lowered motor ability of larvae lacking one ear is rather striking up to near the feeding stage. Thereafter, there is a gradual improvement. Four days after the feeding stage their scores equaled those of normal larvae (FIGURE 24).

The data obtained upon all groups are listed in graph form in the text. The method of study, as presented, gives an objective and quantitative picture of the performance of young larvae under various experimental conditions. It makes possible statistical validation of small differences which might be in doubt if reliance were placed solely upon subjective impressions.

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FUNCTIONAL POLARIZATION IN DEVELOPING AND REGENERATING RETINAE OF TRANSPLANTED EYES*

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STUDIES of the organization and location of the eye-forming centers in the brain wall (Adelmann, 1934, 1936, 1937; Alderman, 1935; Mangold, 1931; Stone and Dinnean, 1943) and the subsequent growth of those centers related to the induction and organization of the lens as shown by Spemann (1938) and many others, reveal how well this sense organ is adapted in amphibians as a tool for approaching problems dealing with polarization during development and differentiation.

The influence of the developing eye cup upon the formation of the medio-lateral axis of the lens has been intimated by the experiments of Woerdeman (1934) and LeCron (1907), but little notice has really been given to it. The outer pole of the developing lens forms the subcapsular epithelium. The inner pole is devoted to fiber formation. The evidence offered by LeCron and Woerdeman seems to indicate that the establishment of this axis may be normally dependent upon the length of time the lens anlage remains in contact with the optic vesicle and cup. When the lens placode was isolated early from the optic vesicle, it formed later only an epithelial vesicle. If the lens anlage was allowed to be associated with an optic cup for a period of time before it was isolated, it could later develop a fiber-forming pole. Even in some of these cases, the fibers would degenerate later. The optic cup is probably exerting a strong influence upon the establishment of this axis. There is a great need for further study of this problem.

Other organization within the lens can also be studied. Early on the anterior (distal) surface of the lens is a vertical suture and on the posterior (proximal) surface is a horizontal one. Focusing attention on the posterior (horizontal) suture, Woerdeman (1934) found that, by rotating the presumptive lens ectoderm 90° , this suture was in a labile form before the period when the neural folds became elevated, but it was determined just before the neural folds closed. Therefore, the polarity or growth direction of the fibers was already predestined at an early stage. According to Woerdeman, this is also borne out by the fact that, when the optic vesicle is rotated 90° , the suture line in the lens is unrotated, although the choroid fissure was rotated.

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Since the direction of the choroid fissure normally coincides with that of the anterior vertical suture of the lens, and is at the same time perpendicular to the posterior horizontal suture, this relationship could be used in further fruitful studies to uncover any influence the eye-forming centers may be exerting on the establishment of the lens sutures.

From the work of Beckwith (1927), Sato (1933), and Woerdeman (1934), one would judge that the time when the choroid fissure itself becomes determined varies in different species of amphibians. Since it can be used as a mark of polarization taking place in the eye cup, and since it marks the groove along which the early *lentis vasculosis* is directed in the higher vertebrates, it is highly desirable that the choroid fissure be examined even more closely than it has been in previous studies.

In the midst of all of these influences for organization, it may occur to the curious-minded that the future functional arrangement in the retina might also be manifesting itself. Therefore, as an interesting challenge from the standpoint of development and regeneration, we shall examine what evidence we have of this example of polarization taking place in the salamander eye.

I have worked for many years on vision in salamanders, where the eye can be successfully transplanted followed by return of vision in the graft. Up to the present time, this has not been done successfully in other vertebrates. We have shown not only that the eyes of larvae (Stone, Ussher, and Beers, 1937; Stone and Cole, 1931 and 1943; Stone and Zaur, 1940; Stone, 1940) and adults can be transplanted to new hosts, and eventually vision returns in the graft, but that the eyes of quite different species of salamanders can be exchanged with equal success (Stone, 1930; Stone and Ellison, 1940 and 1945). This, I might say in passing, has presented an interesting method of studying visual acuity when it is different in two hosts whose eyes are being exchanged. Return of vision can be shown at least four times in the same adult salamander eye (*Triturus viridescens*) repeatedly transplanted to new hosts (Stone and Farthing, 1942). The adult salamander eye can even be successfully transplanted after seven days of refrigeration (Stone, 1946).

When the eyes are transplanted in larval salamanders, very little structural changes take place in the graft (Stone, Ussher, and Beers, 1937; Stone, 1930). The original retina is retained with an occasional loss of a few ganglion cells. The proximal stump of the optic nerve regenerates readily and follows along the pathway of the degenerated distal segment leading into the brain.

In the adult grafted eye of the salamander, a striking difference occurs. Not only all of the optic nerve degenerates but the original retina of the eye also disintegrates quite rapidly, with the exception of a ring of cells at the peripheral or ciliary margin (figures 1-6). From these surviving cells, a new retina is regenerated and, after differentiation takes place, a new optic nerve grows back through the chiasma into the brain, where it apparently makes proper connections. Return of vision is usu-

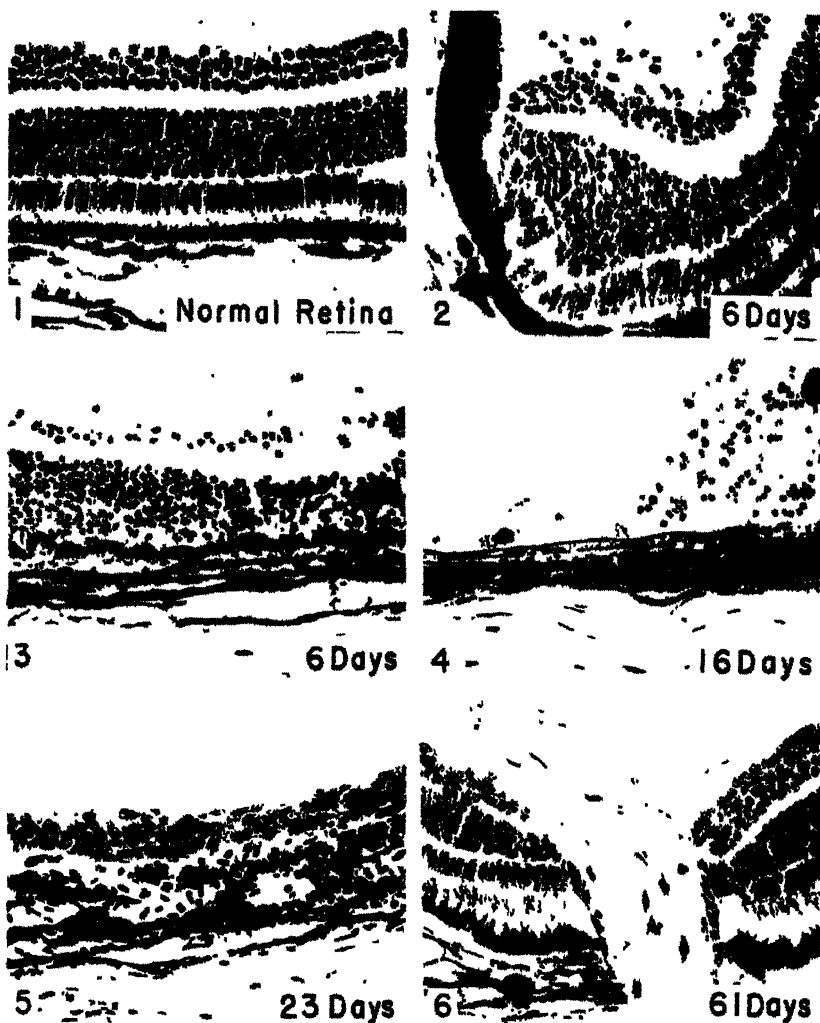


FIGURE 1. Photomicrograph of central portion of normal retina in adult eye of *Triturus viridescens*. $\times 125$. All other figures are from transplanted adult eyes of the same species.

FIGURE 2. Ciliary region in a transplanted eye 6 days after operation, showing far less degeneration than in the central retina (FIGURE 3). $\times 125$.

FIGURE 3. Same case as FIGURE 2, showing extensive degeneration in the central retina 6 days after operation. $\times 125$.

FIGURE 4. Showing complete degeneration of the central retina 16 days after operation. $\times 125$.

FIGURE 5. Showing a regenerating retina, three to four cells deep, in the central portion 23 days after operation. $\times 125$.

FIGURE 6. Showing the completely regenerated retina and optic nerve 61 days after operation. $\times 125$.

ally demonstrated in the transplanted eye between two and a half and three months after operation.

When Sperry, in 1942, first reported reversed visuomotor responses in adult salamanders after optic nerve regeneration in eyes which were rotated *in situ* without removal from the orbit, I examined the effects of various types of rotation on vision in many grafted eyes of *Triturus viridescens* in which the regenerated and new functioning retina could be tested (Stone, 1944). We shall return to these experiments later.

Since we shall be examining the functional quadrants of the retina, we need to know the normal visuomotor responses in the salamander, which are both simple and dependable for our tests. They are demonstrated when the animal moves toward, snaps at, and follows a lure approaching any of the four quadrants in the field of vision. The animal also automatically moves head and body in the same direction with a rotating black and white striped drum only if it passes from the temporal to the nasal poles (postero-anteriorly) through the field of vision of one eye (clockwise for a left eye and counterclockwise for a right eye). These compensatory movements are not elicited when the drum moves in the nasotemporal or antero-posterior direction through the field of vision of either eye.

In attempting to determine the stage in development at which the retina becomes functionally polarized, the right eye was excised, rotated 180° , and reimplanted in embryos of *Amblystoma punctatum* from the closure of the neural folds to a period just before the feeding stage begins. In other words, all the Harrison stages from 20 to 44 inclusive were examined. Vision tests during the larval and adult life of these hosts showed that, up to the late tail-bud stages, the eye could be rotated 180° without normal vision being affected later.

If the eye is rotated around the stage at which the first motor responses are known to appear in the embryo, the visuomotor reactions in the animal later on appear to be confused. Reactions sometimes appear slightly reversed but are not sharp and consistent. It is difficult to analyze them. However, if the eye is rotated at about the time when the beating of the heart is prominent, around Harrison stage 36, a definite reversal in the visuomotor responses will later become evident. The eye is now developed into a well-defined, broad, smooth-rimmed cup. A thin tapetum covers the outer surface of the thick undifferentiated future retina. The lens vesicle is just separating from the source of origin and will soon produce a fiber-forming pole. It fills the cavity of the optic cup against which it has been very tightly pressed ever since it sank inward as a lens plug. Anyone who has tried to remove the living lens anlagen at this time will know full well the implications of these remarks as applied to the operation. If the degree of adhesion with which they stick together is any indication of the importance of events now happening between them before they later separate, it must be all-powerful. In fixed preparations, this relationship is poorly indicated as a distinctive

feature. I believe it is worthy of far more attention than has been placed upon it.

Leading up to this period in development, the functional polarization of the retina is becoming established. Not long after this critical period, all rotated eye cups show that later in larval and adult hosts both the dorsoventral and antero-posterior (nasotemporal) axes are fully expressed by a complete reversal of the vision responses. Whether both axes are established at the same time or at different moments is being investigated by experiments now in progress.

In an earlier part of this discussion, attention was called to the fact that, as soon as one temporarily interferes with the blood supply to the retina in an adult salamander eye, such as transplantation accomplishes, the retina degenerates with the exception of a ring of cells at the peripheral or ciliary margin. These surviving cells aid in the regeneration of a retina which gives off a new optic nerve. This grows out to make connections with the brain, so that return of vision can be demonstrated between two and three months after operation. This offers an unusual opportunity not only to examine the possibility of return of vision but to test whether or not the functional quadrants in the retina can be re-established through the processes of regeneration.

In my studies on many hundreds of transplanted eyes in salamanders, it has been shown that normal vision eventually returns to the graft if it is normally oriented when placed in the orbit. In some manner, the new fibers from the ganglion cells in each of the quadrants in the regenerated retina in adult transplanted eyes register their stimuli with the proper centers in the optic tectum. It is difficult to conceive how normal vision could be re-established if this were not so.

To examine further the functional quadrants of the retina in the adult eye of *Triturus viridescens*, for example, we must perform a few simple operations involving various types of rotation experiments. In the first of these experiments, we shall rotate the right eye 180° antero-dorsally *in situ* and fix it in place after cutting only the conjunctival and muscular attachments. Care is taken to insure that the optic nerve and blood supply are left intact in order to preserve the original retina. To prepare our animal for special tests, we shall remove the opposite left, normal eye. Following our usual technique, the animal, remaining under chlorotone anesthesia, is placed in a cool moist chamber where it continues quiet for 24 hours. During this time, the right rotated eye becomes fixed and healed in place. Animals prepared in this manner are now placed in water in finger-bowl aquaria where they are kept for daily observations. We shall see that the eyes now possess all the quadrants of the original intact retinae functionally reversed.

The animals have a tendency to swim and walk in circles, sometimes in a very excited manner in short circles in the center of the aquaria with the head touching the tail. Their circuitous progression is almost entirely toward the blind side and, as they come to rest, the head con-

tinues to drift for a short distance in the direction in which they have been moving before it is brought back to the midline. When *Daphnia* are swimming about in the aquaria, these salamanders make erroneous strikes at the moving objects. Unless they make contact with their source of food, they may have great difficulty in obtaining it.

In response to a rotating black and white striped drum, the head and body movements are called forth only when the drum passes through the field of vision from the original temporal (posterior) pole to the original nasal (anterior) pole. Since the right eye has been completely rotated 180° in this case, the drum must be moving in the clockwise direction—the one which fails to call forth a reaction if the right eye is normally oriented. Instead of following in the direction of the moving drum, however, the animal walks or swims in the opposite or reverse direction. At first, the head starts drifting in the opposite direction. After reorientation, this nystagmoid movement may be repeated several times. Very soon the animal starts swimming and walking as if pursuing the drum, but always in the reverse direction. When the drum is rotated in the opposite direction (counterclockwise) through the field of vision from the original nasal (anterior) pole to the original temporal (posterior) pole, the animal usually assumes the position of a fixed stare. There is no nystagmoid movement of the head or body, and the animal makes no movements in response to the drum.

A dark object, such as a small piece of red rubber inhaled on the end of a white wire, serves as a good lure for further vision tests. When the lure is brought from in front into the field of vision, the animal immediately seeks it in the opposite direction. When the lure approaches from the rear, the animal moves forward in its search. When the lure is brought above the animal, it immediately darts to the bottom of the aquarium to find the moving object, and when the lure is moved below the glass bottom of the aquarium the animal comes to the top of the water in its pursuit. In other words, all responses are completely reversed. If the animal is anesthetized again and the eye rotated back through the same arc to normal orientation, the swimming reactions and all visuomotor responses to the lure and drum are fully restored to normal as soon as the animal recovers from the anesthetic. We now have sufficient control observations to compare with the results obtained from rotated transplanted eyes with return of vision in a regenerated retina that replaces the original one.

Our next experiment, then, will be to excise a right eye, for example, and then reimplant it upside down. We have then rotated all retinal quadrants of the eye 180° , just as we did in the above experiment when the eye was rotated 180° *in situ* without removing it from the orbit. The general technique of operation is the same as that which I have often described before in the literature already cited. Since the degeneration and regeneration of the retina and optic nerve and return of vision will be completed at the end of two or three months, we must

excise the left normal eye before that time, in order to make our tests comparable to the one above, that is, exclusively on the rotated eye. We find that, as soon as vision has returned, all of the visuomotor responses are as completely reversed as in the case where the eye was rotated in the orbit without destroying its original retina. If the eye is now rotated 180° *in situ* so that its axes are normally oriented, this places the retinal quadrants in normal position, and the visuomotor responses are normal again. Also, if a reimplanted rotated (180°) eye, which has been functioning for a considerable period with reversed vision, is transplanted a second time but normally oriented, the swimming reactions of the animal and the visuomotor responses are perfectly normal in every respect when vision returns again in the retina which regenerates for the second time.

We can study, in the same eye, the effects of rotation on two quadrants of the retina while the other two remain normally oriented (Stone, 1944; Sperry, 1945). This is done in two simple experiments, by excising either the right or left eye and grafting it to the opposite side. If, for example, we excise both eyes, discard the right one and, in its place, implant the left eye without inverting the dorsoventral axis, the quadrants in this axis are normally oriented but the antero-posterior (nasotemporal) axis is rotated 180° , thus reversing only the positions of the nasal (anterior) and temporal (posterior) quadrants. When vision returns in the new retina, the swimming, head movements, and reactions to a rotating drum are the same as when this axis (antero-posterior) was rotated in the eyes reimplanted upside down. Reactions to the lure were completely reversed and abnormal when the lure approached from in front or from the rear, but perfectly normal when it approached either dorsally or ventrally.

In another experiment, if we excise both eyes, discard the right one and, in its place, implant the left eye upside down this time, we maintain the normal orientation of the anterior and posterior quadrants but invert both the dorsal and ventral quadrants. Now, when the vision returns, if the lure is brought into vision above the water in the aquarium, the animal immediately seeks it at the bottom of the aquarium and *vice versa*. In seeking the lure moving into vision from the front or from the rear, the visuomotor responses were perfectly normal. Since this axis (antero-posterior) was not rotated, the head and body movements, swimming, and reactions to the rotating drum were the same as in animals with a single normally oriented right eye. Therefore, we can prove that the functional patterns in each of the retinal quadrants are re-established by regeneration and that the visuomotor responses are guided by the orientation which these retinal quadrants register to the central nervous system. How the peripheral and central connections are properly made to maintain these abnormal vision responses for over three years, is still a mystery and there is, as yet, no good evidence recorded in the literature to help us solve it.

There are other recorded studies of vision responses in amphibians following rotation of functional eyes. Some of the procedures of operation have been carried out in a similar manner and others by a method different from what I have already described. Sperry, in 1943, gave a detailed account of the effects on vision after the adult eye of *Triturus viridescens* was rotated 180° *in situ*, preserving the original retina and optic nerve. Concerning abnormal swimming reactions, drifting head and body movements, the reversed reactions to the moving lure and the rotating drum, my observations corroborate his findings. To determine whether or not the regenerating optic nerve in a 180° rotated eye would connect with the brain in an orderly fashion to show the same abnormal pattern of vision, Sperry (1943a) rotated eyes 180° *in situ*, allowed them to heal in place, and then severed the optic nerve. In some, the cut ends of the nerve were twisted or crushed to increase all chances for confusion as the nerve fibers grew back to the chiasma and brain. In some cases, the blood supply to the retina was apparently not disturbed and the optic nerve must have regenerated without retinal degeneration such as Stone and Chace (1941) found. From his description of the gross appearance of some of the eyes and delay in return of vision, the blood supply to the retina must have been severed along with the optic nerve, in which case the retina was replaced after degeneration. In any event, all of his cases showed vision completely reversed, as in the case of those eyes which, as I mentioned, were reimplanted upside down.

Although one cannot transplant eyes of tadpoles or adult anurans successfully, because the retina will not regenerate (Stone, 1938 and 1940), the optic nerve will regenerate when cut if the blood supply to the retina remains intact. However, Sperry (1944) was able to extend his types of operations to larval and adult anurans by rotating the eye *in situ* and then cutting the optic nerve. The same effects on the visuo-motor responses occurred in these amphibians as had been noticed in the salamanders.

Sperry also made another very interesting experiment. It is well known that, in amphibians, all the optic nerve fibers from an eye pass to the contralateral side. By destroying the optic chiasma and directing the proximal stump of the optic nerve to the pathway of the distal stump on the same side, Sperry (1945) forced the regenerating optic fibers in adult anurans to enter, on the ipsilateral side, the optic tectum with which it never had been and never is normally connected. Peculiar circus movements and mixed reverse reactions and rotating movements of the head and body, accompanied by ocular nystagmus, resulted after vision returned. The animals made errors in spatial localization when striking at a moving object or in trying to escape from an approaching large object. Further study is needed to understand these interesting reactions.

Our objective was to examine evidence dealing with the polarization of the functional quadrants of the retina. However, I think it has become

obvious that the development of polarization within the optic tectum is a very intimate part of this general problem. To unravel it will be no small challenge to our ingenuity.

Summary and Conclusions

(1) Attention is called to the early polarization taking place in both the lens and the optic vesicle and to the interrelating influences being expressed between them at this time.

(2) 180° rotation of many stages of embryonic eyes in *Amblystoma punctatum* demonstrated that functional polarization of the retina is taking place in the optic cup of embryos shortly after the first motor responses appear.

(3) Function was also studied in the retinal quadrants as it was re-established in regenerated retinæ of rotated, grafted eyes of adult salamanders. In some cases, both the dorsoventral and nasotemporal (anteroposterior) axes were reversed (eyes reimplanted upside down). In others, one axis was reversed while the other remained normal (right and left eyes exchanged and properly rotated 180° on the one or the other axis).

(4) Complete reversal of visual perception occurred only in the rotated retinal quadrants, as exhibited by the visuomotor responses to a moving lure and to a rotating black and white striped drum.

(5) The reversed vision is permanent and only restored to normal when the eye is rotated back to normal orientation.

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LOTHAR SALIN

CURRENT TRENDS IN CLINICAL PSYCHOLOGY

BY

A. W. COMBS, H. E. DURKIN, M. L. HUTT, J. G. MILLER,
J. L. MORENO, AND F. C. THORNE



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CURRENT TRENDS
IN CLINICAL PSYCHOLOGY*

Consulting Editor: S. STANSFELD SARGENT

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THEORETICAL FOUNDATIONS OF DIRECTIVE PSYCHOTHERAPY

By FREDERICK C. THORNE

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Clinical Psychology in the Pre-War Period

IN the pre-war period, clinical psychology was in a state of extreme disorganization and individual uncoordinated effort. More than ever, clinical psychologists needed an approach to problems of diagnosis and therapy which was distinctively psychological and not merely a collection of second-hand theories borrowed from psychiatry, psychoanalysis and related disciplines. In the decades since Morton Prince first stimulated the clinical interests of American psychologists, an integrated and original system of clinical psychology had failed to evolve. Texts in the fields of abnormal and clinical psychology were rather sterile and did not contain enough which was distinctively psychological to establish upon it a sound foundation for clinical practice apart from the contributions of psychiatry and psychoanalysis. It therefore seemed desirable to re-evaluate existing theory and to formulate a genuinely eclectic system which, though not original or completely new, would at least integrate the potential contributions of experimental psychology and particularly the psychology of learning. War-time needs accelerated a revision and expansion of the concepts of directive therapy which had been envisaged before the war.

Non-Directive Methods. The decision quickly to publish a series of articles on directive therapy was precipitated by a peculiar situation which had developed in connection with the sudden recognition and popularity of the non-directive technique, as evolved by Rogers.⁴ The impact of the new methods was felt in clinical psychology at a time when there was great war-time need for innovations which would fill the vacuum occasioned by the previous failure of psychology to develop clinical applications which were distinctively its own. As pointed out elsewhere,⁵ Rogers is a very persuasive writer and his zealous enthusiasm caused the new methods to be accepted wholeheartedly and sometimes uncritically. There seemed to be a definite danger that the profession might abandon the hard-earned knowledge of the older directive methods in an impulsive adoption of the new. The danger seemed intensified by the opinions expressed by Rogers in his principal book,⁴ to the effect that older directive methods were now outdated and discredited by the newer "client-centered" approaches. It therefore seemed desirable to make an up-to-date reformulation of directive methods in order to effectively counterbalance too uncritical an acceptance of the non-directive viewpoint.

In contrast with Rogers's non-directive therapy, which appears to represent a rather rigid technical system and which has attracted a group of enthusiastic followers who have formed what almost might be considered a new "school" of psychology, we do not believe that it is desirable or consistent with a genuine eclectic viewpoint to attempt to establish a complete school or system on the basis of the directiveness or non-directiveness of the methods used. In our opinion, directive psychotherapy does not involve a new school or system but merely a more objective evaluation of the methods and indications for utilizing direction in therapy. There appears to be definite value in objectively delineating the clinical relationship in a variety of situations, utilizing eclectically whatever method is indicated in a specific situation. Recognizing that both directive and non-directive methods are valuable in specific situations, the eclectic viewpoint denies the desirability of extremism in either direction. Ideally, a clinician should develop proficiency with all methods which have any valid application to any situation which may be encountered.

Terminological Difficulties. The use of such terms as directive or non-directive to describe systems of therapy is probably unfortunate. As used by Rogers, "non-directive therapy" refers to a specific and sharply delineated system in which extreme non-direction is the most significant characteristic. It is contended that non-directive therapy is a system complete in itself and mutually exclusive of the more orthodox directive methods. Anything less than complete non-directiveness is not considered compatible with being a genuine non-directive therapist. In practical usage, semantic difficulties have arisen from the attempt to apply an "either-or" dichotomy to what is actually a continuum. Rarely is therapy either completely directive or non-directive, and there is a question whether genuine indication commonly exists for extremism in either direction. Most workers are in agreement that non-direction is very valuable in certain situations but do not agree that it is the only and inevitable basis for sound therapy.

Further misunderstandings have developed over the differences, if any, between the "passive" methods developed in psychoanalysis and social case work and the allegedly new non-directive technique. Lowery³ and others have emphasized that the basic principles of non-direction were known and in common use before the work of Rogers, whose contribution seems to have been in carrying them to further extremes than ever before. The principle of being "client-centered" has received general acceptance and is not the sole property of any school. Many of the criticisms leveled by Rogers⁴ against the older directive methods are quixotic in the sense that they deal with undesirable practices abandoned long before by competent therapists everywhere.

In spite of semantic difficulties, the term "directive psychotherapy" was chosen to emphasize that direction does have a legitimate place in

therapy at one end of a continuum of which non-directive methods stand at the opposite extreme. In our own personal practice, we attempt to be genuinely eclectic, utilizing whatever degree of directiveness seems indicated in each specific situation.

The Nature of Clinical Science

The most valid theoretical foundations for directive psychotherapy are derived from a survey of the historical development of clinical science. As pointed out elsewhere,⁷ clinical psychology has a medico-psychological heritage dating back at least to the founding of modern medicine by Hippocrates over 2,300 years ago. The pre-scientific era of medical psychology, in which mental disorders were recognized and treated with primitive religious devotions, has been traced back at least to 5000 B.C. in ancient Egypt.⁹ The significance of these facts is that one must be familiar with the history of medical psychology in order to evaluate recent developments in the proper perspective. There has gradually accumulated a knowledge of mental disorder and its treatment which, at least as practiced by medical physicians, has been taught by word of mouth and precept rather than objectively in books. This statement is true of all the medical specialties, *i.e.*, many details of hospital or surgical practice are not to be found in any book but must be learned in actual experience. This observation is particularly true in the specialty of psychiatry, in which most of the textbooks discuss general theory and omit the details of actual practice. Thus, it happens that there are not yet available objectively oriented, detailed statements concerning what happens in modern directive therapy. Rogers has admirably objectified the formal technique of non-directive therapy, but, as yet, comparable studies are not available for the more complex techniques of directive methods.

Methods of Clinical Science. Clinical science utilizes the same techniques of description, classification, statistical analysis, and explanation as are standard in experimental laboratory science. Operating upon the theoretical foundation of a thoroughgoing materialism, clinical science attempts to discover the causal principles involved in disease and then to discover, by experimental methods, specific treatments for each pathological condition. The fact that description and classification in clinical science have the additional objectives of diagnosis and therapy does not detract from their essential validity, since all steps in the process may be objectively quantified by the same methods of analysis as are applicable to any other type of data. The essential identity of objective description and diagnosis is illustrated by the fact that, the more comprehensive the description, the more complete the diagnosis. It should be clearly recognized that the foundations of modern clinical science rest upon adequate etiological studies which have the objective of describ-

ing, identifying, and diagnosing the causative factors of disease without which rational therapy is impossible.

Diagnosis in Clinical Science. If it is accepted that valid diagnosis is the foundation of rational practice, it follows that some degree of directiveness is present in every clinical relationship, since the clinician must conduct certain examinations to describe the status of the patient. Rogers⁵ has recently denied that diagnosis is either desirable or necessary as a foundation for non-directive therapy, on the grounds that even this amount of directiveness contradicts the basic principle of being "client-centered." Such a viewpoint is in complete opposition to the accepted principles of modern clinical science, which state that valid diagnosis is fundamental to rational therapy. It would appear that even the most completely non-directive technique involves at least two diagnostic decisions, *i.e.*, (a) that the etiological cause of the disorder involves emotional problems which are amenable to non-directive therapy, and (b) that the client has sufficient personality resources to resolve his own problems without directive support. It must be clearly recognized that "blind" or "shot-gun" treatments have long been discredited in modern clinical science even though they may be effective in many minor conditions in which any suggestive method would produce some alleviation of symptoms. The fact that it is possible to treat some relatively superficial conditions by non-directive methods and without adequate diagnostic studies does not necessarily prove that these methods are either scientific or valid in other more serious disorders.

Objectives of Diagnosis. In modern clinical science, the objective of diagnosis involves more than identifying and naming a pathological syndrome. Recognizing that the personality dynamics in each individual case are different, psychodiagnostics has moved beyond problems of classification to the more mature objective of completely describing the etiological factors causative of disorder. Among the objectives of psychodiagnostics are:

- (1) To demonstrate the etiological factors.
- (2) To differentiate between organic and functional disorders.
- (3) To discover the personality reaction of the organism to the disorder.
- (4) To evaluate the degree of organic and functional disability.
- (5) To estimate the extensity or intensity of the morbid process in relation to actuarial data concerning type and severity.
- (6) To determine a prognosis or probable course.
- (7) To provide a rational basis for specific psychotherapy.
- (8) To provide a rational basis for discussing the case with patient and relatives.
- (9) To provide a scientific basis for classification and statistical analysis of data.
- (10) To formulate a dynamic hypothesis concerning the nature of the

pathological process, and the mechanisms whereby therapeutic effects are explained.

Accumulated experience with problems of diagnosis in medical science has resulted in the discovery of a number of basic principles for diagnosis which are presented elsewhere.⁷ Until such time as orthodox methods of clinical practice as developed in clinical science in general are proven to be invalid for clinical psychology, it is our opinion that the clinician has a responsibility for carrying out comprehensive diagnostic studies involving whatever degree of directiveness may be necessary to obtain the desired facts.

Physician-Patient Relationships. A distinctive psychosocial relationship holds in all the healing arts in that here is a situation where one individual expects and demands that another individual shall be more intelligent and have more training and experience. The patient seeks the help of the physician because he has problems which he is unable to solve with his own resources, *i.e.*, he turns to someone who is presumably more capable of achieving a solution. This dominance-submission relationship is present whether the therapy is directive or non-directive. Even with the most extreme non-direction, the patient is submissive in that he seeks treatment from another who presumably dominates the situation even though his method requires that as much responsibility as possible be turned back to the patient. The insistence that non-directive therapy be completely "client-centered" does not alter the fact that the therapist determines what is done and must therefore accept responsibility for the results.

In our opinion, the critical factor in determining whether therapy should be directive or non-directive relates to the decision as to whether the principle of homeostasis can be depended upon to produce a solution of the client's problems. Rogers⁵ emphasizes that non-directive therapy seeks to provide a situation in which the potential personality resources of the client are utilized to the utmost in achieving a solution. Such an assumption may be tenable with minor personality disorders in which the client retains relatively good personality integration and is well enough to regulate himself without direction from without. In more serious personality disorders, however, the client may not possess the resources to achieve adjustment through normal homeostatic processes. In such cases, it is the duty and responsibility of the clinician to use such directive methods as may be necessary to supplement the client's resources.

Sutich⁶ and others have somewhat confused the basic issues by identifying the non-directive method with so-called democratic principles. It is contended that the counselor has no right to undemocratically interfere with the personal integrity and rights of the client. In our opinion, this viewpoint is illogical and irrelevant since it ignores the basic fact that, the more mentally disordered a person, the more irresponsible he

is, and the greater the necessity for regulation by some external agency until such time as the client regains mental health and resumes self-regulation. One of the basic principles of psychoanalysis and other forms of psychotherapy is that treatment is not complete until the patient has been returned to independent living. Clinical practice must be conducted upon the ethical principles of the profession rather than upon ideological systems of political origin.

Legal Responsibilities. Although the matter has not yet come to legal test in the field of clinical psychology because of the relative youth of the specialty, it seems to follow that, as a practitioner of a healing art, the clinical psychologist must be held legally responsible for the ethical and competent practice of his profession according to the accepted standards of time and place. The principles of malpractice in the longer established healing arts are now well set up and are probably applicable to clinical psychology without any basic modification. Having once been approached by a patient for consultation, and having accepted the relationship by entering upon consultation, the physician assumes legal responsibility for his actions and is liable to be sued for malpractice in the event that he is proven to be either civilly or criminally negligent. Errors of omission or commission caused by failure to observe ethical principles or to apply accepted methods of treatment make the physician legally responsible for results caused by such malpractice.

The nature of this legal responsibility probably implies that the physician shall be sufficiently directive to comply with requirements of accepted practice. There are many clinical situations in which the client must be protected from the consequences of his own actions, *e.g.*, when he has suicidal impulses. There are many other clinical situations in which the client does not have sufficient resources to solve his problem and must depend upon external support. Accepting the general premise that non-directiveness is the method of choice where there is high probability that it will be effective, there are a wide variety of clinical situations in which some degree of directiveness is indicated to protect the best interests of the client. From a legal viewpoint, it is assumed that the physician exerts directive control over the clinical relationship at all times unless it can be demonstrated that the patient voluntarily refused to cooperate or otherwise prevented a satisfactory outcome.

Part of the legal responsibility of the physician is concerned with the establishment of a valid diagnosis in order to discover or rule out the existence of malignant pathological processes which might seriously injure the physical or mental welfare of the patient. It is the physician's responsibility to search actively for pathological processes which, if undiscovered, might constitute a basis for the charge of malpractice. Particularly with clients who have seriously diminished personality resources and integration, it does not appear that non-directive methods can be depended upon to effect a diagnosis and adequate treatment.

Therapeutic Considerations

Although therapeutic practice will probably never become as scientifically objectified as is possible in the field of diagnosis, the accumulated experience of clinical science has as valid an application in therapy as in diagnosis. Defining therapy as including all types of formal case handling derived from scientific evaluation of the individual case by competent personnel, rational therapy (whether directive or non-directive) should proceed logically from etiological studies, clinical examinations, and laboratory studies from which a diagnostic formulation results. Depending upon therapeutic indications, methods of case handling may range from the most superficial contacts, as in counseling, to the most intensive depth therapy, as in psychoanalysis.

Modern therapy should be based upon a rigid adherence to materialistic concepts of disease.¹ It is necessary to have a thorough knowledge of gross and microscopic pathology, pathological physiology, and psychopathology in order to comprehend the etiology of the disorders of the total organism. Therapy which is not founded upon the most comprehensive possible understanding of psychopathology cannot be considered to be thoroughly modern and scientific. It is difficult to comprehend how therapy can fulfil the basic requirements of being (a) oriented on the basis of adequate diagnostic studies, (b) directed toward the correction of etiologic factors, and (c) executed on the basis of a detailed knowledge of the limitations of methods unless the therapist assumes responsibility for directing the basic outlines of treatment. Rogers' insistence (⁵, p. 421) that diagnosis may be dispensed with and full responsibility for the conduct of treatment be placed upon the client is not consistent with the accumulated experience of almost all other clinical sciences.

Eclecticism in Therapy. Never in the history of clinical science has there been discovered a universal panacea, or method to end all methods, which is applicable to all types of morbid conditions. A study of the history of clinical science will reveal that the initial enthusiasm which greets all genuinely valuable new discoveries is followed by a period of more sober evaluation in which the indications and limitations of the new method are gradually determined. Thus, the sulfonamides and penicillin were originally believed to have been much more miraculous than later proved true. These comments also appear to apply to non-directive therapy, which is valuable but not the panacea which some have considered it to be.

The principle of eclecticism is the keynote of modern clinical science. To be eclectic is not to identify one's practice with the theories of any one system or "school" of thought. In our opinion, both directive and non-directive methods are valuable when utilized according to a comprehensive knowledge of their indications and contra-indications related to the needs of each specific case.

Directive Psychotherapy. The following outline presents the basic pattern of directive psychotherapy in which the therapist, though client-centered, assumes responsibility for conducting all details of case handling according to the highest ethical and professional standards of time and place.

1. *Adequate diagnostic studies*, involving
 - a. Complete case history.
 - b. Clinical examinations.
 - c. Psychometric and projective studies.
 - d. Laboratory procedures such as electroencephalography.
2. *Making a descriptive formulation* of the psychodynamics of each individual case, including etiology, clinical status, personality resources, and prognosis.
3. *Outlining an individual plan of therapy* with client-centered orientation which is specifically related to the needs of the individual case.
4. *Genuine eclecticism* in therapeutically utilizing all the technical resources, either directive or non-directive, which are available at time and place.
5. *The principles of experimental science* should be utilized wherever applicable at all levels of case handling, and particularly in etiological studies and psychodiagnosis.

In our opinion, this outline of directive psychotherapy is consistent with the historical evolution of clinical science in general and medical psychology in particular. It combines the best characteristics of experimentalism and modern clinical science.

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SOME DYNAMIC ASPECTS OF NON-DIRECTIVE THERAPY

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ALTHOUGH it is only five years since the publication of Dr. Rogers's book, *Counseling and Psychotherapy*, non-directive therapy has captured the attention and imagination of psychologists in a way that has seldom been accorded such an innovation before. At the present moment, it has become a lively and controversial issue in the psychological literature. This vigorous growth and development has been accompanied by inevitable changes in both theory and practice as new elements have been discovered and fitted into place.

Non-directive therapy originally grew out of the experience of many workers dealing with human adjustment and became established as a technique for the very concrete reason that it worked. Faced with the necessity for dealing with clients in everyday work, it is not surprising that the earliest efforts of non-directive therapists should have been turned to the improvement of their techniques and exploring its uses. As a result, the past five years have led us to consistent and well-established basic techniques for non-directive therapy although we are by no means so clear as to why and how these methods operate. We are finding out, however, and some of the dynamic and theoretical bases of non-directive therapy are proving to be no less exciting and far-reaching than the results of therapy itself. It is the purpose of this paper to discuss very briefly some of these current approaches to theory and practice.

The Operation of Need in Therapy. Non-directive therapy is based upon the fundamental principle that *the client not only can, but will, move toward better adjustment when an adequate situation is provided which frees him to do so.* Actually, the concept that the individual not only can, but will, move toward better adjustment is fundamental to therapy of any sort. What distinctly characterizes non-directive therapy in this connection is that it not only recognizes the existence of this drive, but consciously attempts to utilize it for therapeutic purposes.

Non-directive therapists, in discussing their philosophies and techniques, have repeatedly spoken of "the drive of the individual toward growth, health, and adjustment." After the wealth of cases which have now been published, something approaching this drive can certainly be called characteristic of the process. It may even be observed in many cases that the client moves toward a healthy condition even though such movement may be accompanied by the most extreme distress for himself.

Time after time, in the protocols of counseling, the client's own statements reveal his feeling that something approaching this drive is operating within him. For example, one client says at the moment of blackest despair in her counseling:

"I've made a bit of progress here. I'll make more as time goes on, I'm sure. You know, I think sometimes just to get it out of my system will help."

At the very end of the same interview, the client makes this remarkable statement:

"Well, the worst is out—next week we can start reconstructing!"

From the wealth of evidence already accumulated, there can be no doubt that non-directive therapy is often extremely, even dramatically, successful in aiding its clients in a wide variety of problems. When one considers that, in this type of therapy, the counselor has remained "non-directive" and has carefully refrained from the slightest hint of coercion or suggestion, it is not possible for such changes to have been brought about by the counselor. We must presume, therefore, that whatever this motivating force, it has its origin within the organism itself. This is consistent with much of our modern thinking about the nature of the organism. In biology, the drive to maintain self-organization has long been recognized and is described as fundamental to all living things, in the principle of "homeostasis." The very science of medicine has been built upon the concept that the organism can and will return to a state of health if the blocks to its recovery are removed. In view of our present knowledge of the unitary character of the organism, it would be extremely queer if this function were not true in psychological realms as well. We are by no means lacking in evidence that the same function does occur in the realm of psychological processes, for it is a well-known observation that many of our mental patients get well without outside assistance and even, sometimes, in spite of assistance. We might describe this need, as it operates in psychology, as a need to maintain or enhance the individual's personal organization.

One might raise the question at this point that, if the client has a drive toward health, if he can and will move toward adjustment, why is therapy ever necessary? The answer seems to be that sometimes the organism's need to maintain or enhance its personal organization is blocked by forces preventing its maximum fulfillment. Such blocks may arise, externally, in the individual's environment, or, internally, within the individual himself.* In either case it is likely to result in confusion and failure of accurate perception. This, in turn, results in random behavior, or what has been called "escape behavior," offering only a temporary solution to the individual's problems, and, more often than not, even complicating them further. By giving the client temporary relief and making it possible to protect his organization for a time, "escape

* It will be recognized that this is true in medicine as well, wherein disease, defined in its broadest sense, is a state in which normal bodily processes are obstructed by either external or internal attacks upon the organism's organization.

behaviors" block more adequate perception in terms of which a more permanent solution might be achieved. Thus, confusion and distortion of perception play a large part in the production of the individual's maladjustment. Being unable to differentiate his situation clearly, the client may be unable to find an adequate solution and so continue his maladjustment indefinitely.

But another factor enters the situation which further prevents the free activity of the drive about which we have spoken. This is the effect of threat upon the individual and his self-organization. In addition to the confusion and failure of adequate perception in maladjustment, it will be recognized that one of the most commonly described characteristics of maladjustment, and particularly of neurosis, is the feeling of fear expressed in one form or another by the subject. Oftentimes, the object of fear is vague and undifferentiated. Such feelings of threat are characteristic of situations in which the individual is blocked from achieving the satisfaction of his fundamental need to maintain or enhance his self-organization. What is more, the closer such blocks come to affecting the organization of self, the greater the severity of the threat felt by the individual and the more active become his attempts to find a solution to his problem. Feelings of threat have unfortunate effects upon the client's ability to make a satisfactory adjustment.

We have stated previously that the basic need of the organism is to maintain or enhance its personal organization. When this basic organization is threatened, however, the organism has no choice but to defend the organization which exists, so that a movement toward a more adequate organization becomes impossible. Indeed, there is even danger that, if the threat is great enough, the client may be driven deeper into his present organization and his maladjustment made greater. This effect of threat has not been given much consideration in psychology although it is well known to the layman and the advertising expert or salesman. It is common observation, for instance, that "nobody ever wins an argument," and the quickest way to get a man excited is to threaten the things he holds most dear, usually himself. We may even hope that, some day, our diplomats will come to recognize this principle of human relationships. In our own science, we have long recognized the principle that "aggression yields aggression," but too often we have given mere lip service to the idea and in applied psychology have often acted as though it never existed.

We have seen in this theoretical discussion that the individual can and will move toward improved self-organization, but that this is often prevented by the client's own confusion or failure of adequate perception and feelings of threat which hamper or preclude movement toward new self-organization. It would appear, if this analysis is correct, that if we can remove an individual from threat and assist him to differentiate his personal organization more clearly, he should be free to move toward a more adequate and satisfactory self-organization and hence to better

adjustment. This is precisely what non-directive therapy attempts to do. It consciously creates a relationship between therapist and client which scrupulously protects the client from threat, while, at the same time, encouraging and assisting him to a clearer and more accurate differentiation of his self-organization and its relationship to the world in which he moves.

Elimination of Threat. In his counseling relationship with his client, the therapist attempts to eliminate threat by an attitude of sympathetic understanding and acceptance and a careful avoidance of any action which might be construed by the client as a violation of his integrity.

In the early days of non-directive therapy, a great deal of stress was placed upon counselor techniques by means of which the individual was thought to be helped to his adjustment. Recently, however, there is a growing feeling among therapists using this approach that the atmosphere of the counseling relationship is of even greater importance than techniques. By this atmosphere is not meant the physical surroundings or the prestige of the counselor, but a "permissive atmosphere" consciously created by the counselor in his relationship with the client. In the therapy session, the client finds himself in a situation characterized by warmth and responsiveness on the part of his counselor, free from pressure and coercion of any sort, and in which he may express himself in any way he pleases within the very broad limits of the counseling relationship, which Rogers¹ describes as follows:

"From the client's point of view, while he may not be conscious of these elements at the outset, he does respond to the atmosphere of freedom from all moral approval or disapproval. He finds that he does not need his customary psychological defenses to justify his behavior. He finds neither blame nor over-sympathetic indulgence. He finds that the counselor gives him neither undue support nor unwelcome antagonism. Consequently, the client can, often for the first time in his life, be genuinely himself, dropping those defensive mechanisms and over-compensations which enable him to face the world in general."

As Rogers has suggested, the therapy situation is probably vastly different from any the client has ever before encountered. In daily life, the person who desires to tell others of his problems finds others all too ready to tell him theirs. Even worse, he is likely to be subjected to attempts on the part of those around him to force change on him in some fashion or other. This does not occur in non-directive therapy, however, and the client usually recognizes and makes use of the opportunity provided him. Note how the following clients express their understanding of this "special" kind of atmosphere created in the therapeutic relationship.

One client says:

"When I'm talking to you, I just can't lie to you. Why, I can't even lie to myself. I don't think I've ever been so honest with myself before."

Another painfully shy young woman, extremely frightened by men, tells her male counselor:

"In here I can talk to you. But if I were to meet you out in the hall you would be just another man to me and I couldn't talk to you at all."

The same client writes two years after her experience in counseling:

"My counseling experience came at a time when my life was most disorganized. I went to you as a last resort. It was distasteful to talk to anyone about my problems—but the more I talked and the less you seemingly interfered, the better I felt and the more clearly I saw what the trouble was. Since that experience, my life has become more positive and creative. I have gradually placed more confidence in my own abilities and opinions. I feel completely happy and organized most of the time. I feel at ease with people of both sexes and all ages and have no trouble in meeting or liking the hundreds of people I have come in contact with on this new job."

In such a sheltered atmosphere, protected from the necessity of self-defense, the client finds himself free to examine himself in any way he desires. He finds no blocks to free expression placed in his path, and can utilize the therapy session in his own way as his needs determine.

It may be argued that no counselor worth his salt goes about threatening his clients. Experience has shown, however, that even the best-intentioned counselor cannot avoid threatening his client when he uses directive techniques. Even so simple an act as answering a knock at the door may be construed as threatening by the client, given the proper circumstances. This has actually happened in our experience. After answering the door and briefly directing the inquirer to another office down the hall, the counselor returned to his client. Note the feeling of threat implied in what the client said next:

S.—"You know, I thought when you went to the door, you would tell me that's all for today."

C.—"You thought I really wasn't interested enough to go on today, is that it?"

S.—"That's right. I know it's silly, but I find I am very sensitive to anything you say or do. I'm so afraid you don't really want to help me. I'm afraid you might think the things I have to say are silly and I keep expecting you to tell me something that will upset me more, like the others I've been to—Oh! Don't misunderstand me!—I know by now that you won't, but I still get panicky inside when I think you might not want to help me."

It must be recalled that it is the client who feels threatened and not the counselor. The threat to organization lies not only in the actions or words of the counselor but in the peculiar meanings of these behaviors to the client. What is more, since these meanings exist within the client, they are not open to observation by the counselor but lie outside his control. Frequently, the client may feel threatened without giving the slightest sign of this fact to the counselor. Most clients have learned, through long experience, how to keep their feelings from being observed, but even if they had not, it is too late to deal with the effect of threat after it has occurred and the client has reacted. Severe threats to the client may even in some cases be sufficient to prevent him from ever attempting to examine a particular line of thought once he feels this is inappropriate

or would be disapproved by his counselor. To avoid such threats consistently is by no means easy, as many a beginning counselor has discovered.

As we have suggested in a previous paper,¹ protection of the client "makes possible the pursuit of a question to its ultimate conclusion, a process greatly impeded by directive remarks." This freedom to examine and to pursue a line of thought to its "bitter end" makes insight possible with greater speed than is otherwise practicable. As one client put it: "It's like clearing away the brush that confuses the path." Freed from the necessity of defense and able to carry out such "bitter end" analyses, it becomes possible for the client to explore his field of meanings in any way he desires and to arrive, eventually, at a clearer differentiation of his self-organization and his relationship to the world in which he operates.

Aiding Differentiation. The second major function of non-directive therapy lies in assisting the client to more adequate differentiations. As we have seen, maladjusted clients characteristically are confused and have failed to differentiate clearly the various aspects of their self-organization and the environment in which they operate. Unable to differentiate clearly the various aspects of his field, the client in trouble finds himself anxious, worried, and vaguely afraid of something he cannot quite put his finger on. So long as this situation continues, a change in self-organization seems very unlikely. Indeed, it is likely to be very nearly impossible.

To assist his client to make such differentiations, the non-directive counselor does two things: (1) he encourages his client to express himself freely and in any way he pleases; and (2) he assists him to examine his personal meanings through a technique known as "recognition and acceptance of feeling." From a theoretical point of view, we might just as well call this technique recognition and acceptance of personal meaning, for that is essentially what it is. Feelings, after all, are simply the client's way of expressing the meaning of a situation for him. They express his personal reaction to the situation he is describing.

Personal meanings are crucial in the client's behavior, for we behave not in terms of events but in terms of the meanings of events for us. If Mrs. Jones, for example, feels that her husband is a brute and a heel, it makes little difference in her behavior whether he actually is or not. If she feels that way, it is enough. What is more, pointing out to Mrs. Jones that her husband is really not a brute but a very fine fellow will be of little help to her since this is not her opinion but the therapist's, and may only serve to demonstrate that the therapist "just doesn't understand." It will be recognized, too, that such a statement by the therapist constitutes a threat to Mrs. Jones and may force her to defend her position more tenaciously than ever. Telling the client is of little value if he cannot accept such information into his personal organization of

meanings. It is the personal meaning of facts which motivate behavior, not the facts themselves.

It is the very fact that the individual's personal organization of meanings is unacceptable to others that classifies him as maladjusted. In adjustment counseling, therefore, some reorganization of these personal meanings must occur if therapy is to be truly effective and if any change in behavior is to result. Thus, the counselor in non-directive therapy assists his client to explore his personal meanings by recognizing and accepting those meanings as they appear. This helps his client to explore his personal organization of meanings further, helps to clarify them and holds them up for further examination. Under this kind of treatment, the client is able to make greater and greater differentiations in his meanings, resulting eventually in a new organization of personal meanings, as a result of which change in behavior becomes possible. The following excerpts from a single interview show this kind of change of personal meanings in progress.

Mrs. Brown came to the counselor extremely upset over her family relationships and considering the possibility of a divorce from her husband whom she described as a completely intolerable person. After spending some twenty minutes in a veritable tirade against her husband, she made this remark:

"But you know, other people don't see him this way. They think he is a grand fellow and a good father and all that, BUT" and at this point she launched again into a further broadside assault on her husband, his family and everything connected with him. When this had subsided, she said, musingly:

"But he does remember my birthdays and things like that. You know, he has never forgotten once." This was followed by more to the effect that her husband was distinctly not all he might be.

A few moments later, this feeling was expressed with a rather self-conscious giggle: "Golly! I've really been pretty hard on him, haven't I? He really does have *some* good points, you know." A few minutes later, Mrs. Brown says further, "You know, I've lived with that man for ten years now and he still doesn't hang up his pants"—then a long pause as she goes on to say, "But you know, I really think sometimes I kind of like it, looking after him," and she closes the interview with this remarkable statement:

"I guess the real trouble is with me. I'm beginning to think I need this more than he does."

By the use of the technique of recognition and acceptance of feeling the counselor aids his client to clearer and clearer differentiations of the meaning of things for him, while at the same time avoiding any threat to his client. By recognizing and stating these meanings clearly and sharply, he assists his client to further differentiations until, eventually, this process may arrive at those differentiations most troublesome or fear-producing for the client. Sooner or later, the client reaches this point in spite of himself. Once it has been reached, new adjustments become possible.

It may be argued that, if the emphasis of therapy should be upon the meaning of events to the client, then interpretation, questions, directions, and the like should help to speed this process along. It would ap-

pear that, if the therapist can perceive these meanings and point them out to the client, the client's differentiations should occur more rapidly. Actually, such techniques may often be disastrous to the client's progress and may impede or even destroy the counseling relationship. Interpretations and information given when the client has not reached a stage of differentiation where he can accept such interpretations are not *his* personal meanings, but his counselor's. As the counselor's interpretations, they may even appear to the client as threatening and force him to defend his position not only against the world at large, but the counselor as well. This failure to accept information into his personal organization is well illustrated by our own life situation wherein we often know what we *ought* to do but don't do it. Until a concept has been accepted into our personal organization, it has little effect on our behavior.

In the permissive atmosphere created by the non-directive counselor and aided by the therapist's techniques of recognition and acceptance of the client's personal meanings, the client is able to differentiate more and more clearly the various aspects of his personal organization. In this process, he is exploring and defining the relationship between himself and the world in which he moves. As counseling progresses, he comes to differentiate more and more clearly the situation in which he is operating, the source of the threats which he has felt, and finally is able to arrive at a new organization of personal meanings resulting in behavior less at odds with the society in which he must operate and infinitely more satisfactory to himself.

A minister's daughter had led an exemplary life for the benefit of her father and his congregation. When she was forced to live her own life in college, away from her family and community, she became deeply confused and upset, and finally came to a college counselor for help. Note, in the following excerpts from counseling, how she struggles with her concept of herself and her relationship to the world about her:

"A couple of years ago I heard one of Dad's sermons in which he said 'a person has to like himself.' From then on I took it for granted that I did like myself. I decided that I wouldn't change for anything—until this week when I began wondering if I really did. I decided I liked myself but I also despised myself."

"I've decided I'm a two-sided, two-faced person. I've always had to act one way although I felt another. I've always had to be something I'm not. I give appearances but, down under, I'm not that at all."

"I'm not sure what I'm like—I don't know what I am. I'm a man without a country. What is myself? It's funny how sure I was and now I'm not sure at all. I'm afraid of everything at the moment, but I can't find what it is I'm afraid of. I'm afraid to live like this for the rest of my life, but I'm even more scared as to what to do about it. I'm afraid even to think about it. The more I think, the more scared I get."

"My problem is myself. What am I? I'm human, female, five foot seven, period. I want to be sure, but I'm not even sure of myself. Maybe I know what I am but I'm afraid of it. I'm in a panic about myself."

"I never felt I could be myself. I couldn't because of father's job. Now I know I must change myself, but what am I? I must know that."

"It's a battle between what I think I am and what I really am!"

Another client, a mother who had been rejecting her children for several years, arrives at this differentiation in counseling:

S.—“I’ve been struggling with this thing for weeks but I know now what it is. I’m ashamed of myself for not seeing it before and ashamed of myself because that’s what it has turned out to be.”

C.—“The thing you have been afraid of is pretty clear to you now.”

S.—“Yes, it is, but I can’t say I’m proud of it. All along I’ve been upset because of what other people’s children were like. My poor kids—when I think of what I’ve done to them!”

C.—“You feel pretty upset by what other people think.”

S.—“That’s right. Always it’s been like that. I’m afraid I’ve been pretty selfish in all this.”

The Reorganization of Self in Therapy. It is interesting how little the re-evaluation of the self in non-directive therapy has been recognized. Some writers have even gone so far as to suggest that, until non-directive therapy found ways of changing the self concept, it could not truly be considered a fundamental form of therapy. Actually, it is this very redefinition of the self which is the most striking and characteristic aspect of the entire process. Raimy,³ in his doctoral dissertation, has clearly demonstrated that shifts in the self concept do occur in non-directive therapy.

With a clear differentiation of the relationship between self and the environment about it and with clarification of the nature of the threat to its organization, the stage is set in non-directive counseling for a shift in the self concept. Indeed, such a shift in the concept of self becomes not only possible but almost inevitable. The need of the organism to maintain or enhance its personal organization forces the client to a reorganization of self when it becomes clear that such a reorganization works to the enhancement of the organism involved. The effects of this in non-directive therapy are among the most exciting and fascinating aspects of the process. Since the individual’s behavior is a function of the meanings he has given to himself and the world about him, we would expect that changes in the self concept would be accompanied by considerable change in the behavior of the client as well. This is exactly what occurs, in many cases, following non-directive therapy.

One client says near the end of her counseling experience:

S.—“There I was, sitting in the library reading. All of a sudden it hit me. It was the craziest thing—just like that. I thought, ‘It’s stupid of me to go on like I am. I’m just not a brilliant person and that’s all.’ It was just all me—mentally! It was so funny. There I was—and I thought, ‘Here everybody else is adjusted to themselves and you’re trying to adjust to everyone else but yourself.’”

C.—“You feel it’s necessary to accept yourself.”

S.—“That’s right. I know I’ll never change from what I am. I was so excited, I couldn’t think last night. I thought—*Suppose you had told me what to do?—I’m so glad you didn’t!*”

Another client who illustrates this change in behavior was an extremely masculine-appearing young woman who obviously prided her-

self on this characteristic. Raised in a family in which her brother got all the attention while she was forced to run errands for him, and with no one to play with but boys, the onset of puberty was deeply resented. When an attack of infantile paralysis left her further unable to compete with boys, she set out to prove that she could. She made herself proficient at all kinds of sports, but was never able to achieve an ability equal to a man's. In college, when other girls were looking forward to marriage, she resented her figure and femininity. As she expressed it, "I just can't see the boys I go out with as potential husbands. Pals? Sure. Husbands? No!" She talked in a deep and husky voice, and would rather be seen dead than in anything but slacks and a shirt. In the next-to-last interview held with this young woman, she came to a different organization of her self concept and accepted her role as a woman. She states:

"You know, I've been trying this weak and helpless angle, and it works. I'm beginning to think I like it. Why shouldn't I? After all—I *am* a woman. I can't be like my brother, so I'll be like me. I'll just have to accept myself as small. I can't do everything—I feel like being smooched with but I don't have anyone to do it with. But that will fix itself. —I'm beginning to feel important!"

Next time the counselor saw this woman he was honestly astounded at the transformation. He hardly recognized his client. She looked feminine from head to toe! Perhaps the best way of expressing this change may be observed in the client's own statement:

"I'm more feminine now than when I first came in. I feel more feminine and I guess I must act so, because lots of boys that never did before want to help me now. They even want to kiss me and carry my ski poles for me. John especially. I think he likes me better and I know I like him better too—I think I must be on the right track. I feel more comfortable—very comfortable—and—I like myself better too."

The Client-Centered Nature of Non-Directive Therapy. One of the most frequently misunderstood aspects of non-directive therapy is the non-directive therapists' use of the term "client-centered." Some writers^{2,6} have inferred that, since non-directive therapy claimed to be "client-centered," by implication all others were not. This is a most unfortunate interpretation. Certainly, any counselor of whatever philosophy, if he is worthy of being called a counselor at all, is interested in his client's welfare. The term "client-centered" as used in non-directive therapy, is not meant to apply so much to the counselor's concern about his client's welfare, as to the way in which the counselor attempts to see the client's problem *through the client's eyes*. We have repeatedly stressed, in our discussion, the importance of the client's own meanings of events. It is the counselor's concentration on and attempts to understand these personal meanings that is meant by "client-centered." For example, Rogers⁵ states, "As time has gone by, we have come to put increasing stress upon the 'client-centeredness' of the relationship, because it is more effective the more completely the counselor concentrates upon trying to understand the client *as the client seems to himself*." (Italics as in the original.)

This counselor task of seeing the client and his relationship to the world about him as these appear to the client himself is no mean feat. It is one thing to understand this technique; it is a very different matter to put it in practice. The beginning non-directive counselor is very likely to discover, early in his experience, that the seemingly simple non-directive principles become intricate and difficult when the attempt is made to put them to work. He soon discovers that what he knows is of far less importance than what he is. Indeed, much academic knowledge usually required of the psychologist is unnecessary for effective non-directive therapy. This idea has disturbed many critics of non-directive counseling. They have been deeply concerned at what appears, at first glance, to be a renunciation of training. Actually, while it is true that many diagnostic and statistical skills are not essential, effective non-directive therapy requires of the skilful practitioner experience, sensitivity to people, understanding, self-discipline, and a personal growth and development by no means easy to arrive at. While academic training alone may contribute to understanding, it is no guarantee of an effective non-directive therapist. Knowledge of facts can be readily grasped by an intelligent student, but personal growth and self-discipline can only be achieved by most of us through arduous experience.

Recognition of the individual and an absolute respect for his integrity is not just an idea in non-directive therapy. It is a working principle.

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THE THEORY AND PRACTICE OF GROUP PSYCHOTHERAPY

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JOHN LEVY's Relationship Therapy as we have crystallized, developed, and applied it to groups, is direct interpretative therapy. It is geared to go as deep as but no deeper than the patient's needs and capacities indicate. It is not meant to be palliative, nor to strengthen the patient's repressions.

In the course of a lifetime, people build up various defenses against certain unacceptable instinctual drives, such as hostility. When these defenses are not working well, they become anxious and develop neurotic symptoms (which are danger signals like any symptoms of a physical disease) or they experience general anxiety. This often brings them to the psychotherapist for help. In treatment, they make another attempt to keep away the unbearable drives. Their defenses become *resistances*, and the therapist must dissolve them one by one until the basic drives can be reached, re-evaluated, and handled in a more mature way. This process will strengthen the patient's ego and bring about a real character change.

The Relationship Therapy is a transference therapy, psychoanalytically oriented. Like any such therapy, it is based on the premise that, sooner or later, the patient will bring into his relationship with the therapist the full range of his emotions—his fears, resentments, demandingness, tenderness, and so on. He will express them in the characteristic attitudes and patterns which, under pressure of his early familial experiences, he has built up to defend his ego. For example, as a child, the patient may have been afraid to show his resentment to a tyrannical father. He may have discovered that one way to escape his dilemma and find temporary relief was to run away—physically or symbolically. He will do the same in the treatment situation as soon as resentment is aroused toward the therapist (as a person of authority). Unless the therapist is keenly alert to the first signs of approaching hostile feelings and brings them into the open, he is likely to lose this patient.

Let us examine another similar but more complex example. I was working with a young man who had felt rejected by his mother, toward whom he had a strong attachment. His frustration filled him with rage, but he dared not be angry lest he lose the little he got from her. Both his erotic and his hostile feelings had caused him so much pain that he tried to avoid them. He had started psychotherapy twice and twice he had run away from his woman therapist as soon as he became aware of her as a woman. He left enraged notes behind accusing her of seducing him. It was a kind of triple defense. He projected his erotic feelings on to her,

thus getting rid of his anxiety about them. Then he converted his rage at expected frustration into righteous indignation at her bad behavior. Finally, he rid himself of the whole anxiety-producing situation by running away. His people, however, insisted on treatment and he was sent back, this time to me. As I expected, he entered treatment easily, talked with apparent frankness and soon claimed improvement. When he expressed inordinate gratitude, I said, "You know, when you feel so grateful to me, you are apt to become very fond of me, and if this happens you are going to be pretty upset. You may even want to run away." He denied it, but stammered and blushed furiously. I went on, "Patients have all kinds of disturbing feelings toward their therapists. But it is different in treatment from outside, and you will find it is good to talk about these feelings here. This is the way I help people." The boy managed to stay and, instead of running away, he eventually described how he would like to marry me. Again I warned him that, on finding that I have to be impersonal, he would be angry with me and want to leave. Again he was able to stick it out and I was soon listening to a description of how he would like to murder me—with an ice pick. Treatment scenes are seldom as dramatic as this, perhaps never in a group, but the principle is the same.

As you can see, the pivot on which this therapy moves is the analysis of every implication of interpersonal attitudes as they near the threshold of consciousness (whether positive or negative). Trivial as these first evidences of irrational feelings may seem, they are the clues to the patient's ego defenses and, in treatment, these defenses become the resistances. Dissolving the resistance at the earliest possible moment speeds up treatment.

Each patient has any number of such defensive patterns, and, as the therapist exposes one after another of them, the patient gradually comes face to face with the unbearable impulses which were originally behind them. Recognizing them in the controlled interpersonal setting helps him to become aware that they have little bearing on the immediate situation, and to discover their real meanings and sources. Once that has happened, he becomes free to relate himself to people in a more realistic way.

Modification of the feelings of guilt derived from identification with a permissive therapist and the catharsis of dammed-up feeling are a part of therapeusis, but we feel that the greatest change in the patient comes from the gradual dawning of insight within himself as he is helped to recognize how inappropriate his neurotic attitudes are. He catches himself in the act, as it were. The essence of the treatment, therefore, is in the living experience of the relationship.

Since we see our patients only once or twice a week, we do not let them struggle alone with the anxiety they must experience as they become aware of their instinctual drives. We help them to verbalize the anxiety-ridden feelings as these reach the threshold of awareness instead

of waiting until the patient is fully conscious of them. This requires skilful timing; for interpreting too soon will *give* him the insight which should *come* from within and will make an intellectual process of treatment. It may succeed only in strengthening his resistance. Waiting too long to interpret, on the other hand, will increase the patient's anxiety between visits when the therapist is not available for help. It would also increase the length of treatment considerably.

These are the principles of therapy, and they remain the same whether used with individuals or with groups, with adults or with children. In the group, the therapist finds himself handling many relationships at once; the feelings of the four or five women (or children) toward one another and toward himself. These start as surface reactions but gradually develop into transferences. The same kinds of defenses and resistances display themselves as occur in individual treatment, and, if one uses a transference therapy like this one, much the same kind of treatment process takes place as in individual therapy.

There are, in addition, many intra-group impacts of which the therapist must be constantly aware and which he must handle. Some women, for instance, express one kind of feeling or another more easily than others and act as catalytic agents for a time. Others will take over, as it were, when other kinds of emotion come up. Although the patient-therapist transference may not become as intense as in individual therapy (sometimes it does), the subjective attitudes of the other patients serve rather quickly to bring into being what we might term secondary transferences.

For example, Mrs. B. may display attitudes that remind Mrs. A. of her mother or, for that matter, of her father. This may arouse tender or resentful feelings toward Mrs. B. which will be expressed in Mrs. A.'s usual patterns. The therapist must interpret such relationships just as he does when they apply to himself, and he must also handle the feelings of the recipient of such transferences. Among such feelings will almost certainly be sibling rivalry, for the group naturally tends to enhance this feeling, which comes out more quickly and realistically than in individual treatment. If the therapist has a tendency to steer away from socially unacceptable feeling, the group will soon develop a sewing circle character. If he sits by, passively but permissively, some super-ego modification and some catharsis will occur. If he interprets skilfully, timing his comments so that the patients themselves become aware of their conflicts and anxieties, real character change is possible in the group, as in individual treatment. To reach this goal, the therapist encourages all emotional expressions, however socially unacceptable. He helps to bring them out by repeatedly bringing to light the undercurrents of feelings as he senses them behind the factual productions of the patients.

The nature of his comments, as well as his timing, must be geared to the patients' level, psychologically, educationally, and socially, so that

they can accept and assimilate what he says. If what he says is too strong for the patients' weak egos (if he deals narcissistic blows), treatment will be vitiated. It follows inevitably, then, that if the therapist's own drives enter into his work beyond the point at which he can recognize and control them, he will create trouble instead of bringing relief. Supervision during training will control this possibility. There is another safeguard, however: such a therapist would soon find himself without any patients—certainly without any groups.

Perhaps the best way of illustrating the method is to try to show it in action by describing a particular group. In order to simplify this too ambitious undertaking, I shall try to follow some of the more illuminating moments in the progress of a particular patient, Mrs. S., within her group, bringing in the others just enough to show the kind of interaction that takes place.

The group I have chosen to discuss consisted of four women who met once weekly. They were mothers whose children were also in treatment at the New Rochelle Child Guidance Center. One patient is an attractive woman of about thirty-four who came because her older child, a girl of seven, suffered from extreme shyness, enuresis, nail-biting, and general irritability. The mother is a neat, modestly dressed woman whose most noticeable characteristic was an almost constant artificial smile which gave her face a mask-like quality.

On the day of our initial meeting, Mrs. S. was the first to arrive and immediately began to complain about her husband and his mother. She spoke stiffly and without feeling, as if reciting a lesson. Her husband, she said, like his mother, never praised but often criticized her, especially for being extravagant. She felt this was unfair because she had always had to be thrifty by necessity. Although her mother and her aunts had been hard up, they were always generous and did not stint with praise. In spite of her righteous indignation, however, she felt that, somehow or other, the whole thing was her fault anyway, perhaps because she was no good as a housekeeper or a mother and did not know how she could ever improve. Since we are, on the whole, passive during the first interview, my only comment was an attempt to help her feel I understood the way she felt. I interpreted not the material, but the undercurrents of feeling, saying only, "I get the impression you have been trying for a long time to keep your chin up." She burst into tears, and spoke with less tension after that.

When the other group members came in, Mrs. S. went right on addressing herself to me alone for a minute. Then she apologized for taking too much time. She was not ready yet for an interpretation of the sibling rivalry she revealed, nor of her need for approval. I waited for an occasion when it was so clear that she herself would see it.

For the rest of the hour the women talked about their children's behavior, as they usually do before they have accepted the idea that they themselves are patients. Mrs. S. listened for the most part, but occa-

sionally she gave an example of similar behavior—however, always choosing it from her own childhood.

Since first interviews are usually revealing as a kind of forecast of things to come, it is a good idea to ask, "What does this all add up to?"

(1) Mrs. S.'s artificial, tense manner seemed to indicate that she was struggling to keep an overwhelming anxiety out of sight.

(2) What she said seemed to imply, "I am a child, and I'll be good and tell you what you want to know, but please be kind and approve of me."

(3) Her disturbance over being criticized made me guess that she used approval and praise to cover up some deep-seated anxiety and warned me that she would want praise from me, too, which would serve to build up her defenses; that she had come for treatment for that very purpose and that when it was not forthcoming she would feel criticized, rejected, angry, and anxious.

(4) Her disregard of the other women at the beginning and her quick apology for it revealed her strong drive to keep the therapist (as a mother person) to herself and that one of the first things to handle, as often happens in a group, would be sibling rivalry.

(5) Her use of examples from her own childhood confirmed the feeling that she still regarded herself as a child. What had prevented her assuming adult responsibilities would be left to find out. It might also shed light on the deep sense of inadequacy expressed in her inability to cope with household and parental duties.

Since Mrs. S. had not been aggressive enough to keep the floor at the first meeting, I was not surprised when she came late the next time. We find it a useful rule to comment on such evidences of hidden feeling. I said, "I think it was a little harder for you to come this time." She denied it, referring to the silly use of this interpretation in the social-work school she attended for a while. I did not press the point because we feel that arguing with a patient implies self-defense and might make her feel that aggression or resentment is not acceptable. She might, then, shut off future display of it, whereas we want her to feel free to express any feeling whatever. I said only, "You must have been disappointed in the group last time because you had so much to say and so little chance to say it," whereupon she answered, "Well, I did have a lot I wanted to talk about." I knew all the women would be experiencing rivalry at this time, so I said, looking around, "Naturally, every one of you would prefer to be seeing me alone." I could tell by their expressions I had hit home. I said this to relieve them of the need to hide this aggressive feeling, and so to pave the way for later, more explicit expression and interpretation of sibling rivalry. The immediate result in the second interview was that, when Mrs. S. spoke again about her husband, she could include the other women instead of looking at me alone. Months later, when one of the women told how jealous her two boys and husband were of her ministrations, I compared it to their group situa-

tion. By this time, they had become aware of their rivalry and could see it in this new sense.

Mrs. S. was early for the third interview and announced that she had made an inexcusable display of herself the week before, when she "blew up" at me. I said that apparently she had felt criticized by me. She said, "I always felt criticized at school when they made that interpretation," and she told us about a teacher who had told a girl to stay away altogether as long as she felt so resistant that she was always late. I said, "I have an idea you are afraid I might send you away too." Mrs. S. nodded, her eyes filling with tears, as she told us how she had always felt on the fringe of her family as a child—lonely and lost. She seemed freer now and dropped the stiff, artificial manner. When she talked about her husband and mother-in-law again, she gave vent to strong resentment.

Apparently, she gained some relief from this tirade, for she soon reported that she was getting along much better with her husband. She had, for the first time, been able to confide in him her feelings about his mother and he had actually taken her part and had been much sweeter to her. She also made him see how she felt about treatment, to which he had been antagonistic. Such immediate results from the first six weeks or so of treatment are not at all rare, for the first layers of anxiety are lifted off. Sometimes the exigencies of the case lead us to terminate treatment at this time, but if the patient needs it, can profit by it, and we have time, we go on.

Mrs. L., a thin, determined-looking woman, who expressed aggression under a usually pleasant, soft-spoken manner, was the woman in the group who had the greatest impact on the treatment of Mrs. S. She was of a lower socio-economic and educational level but had, at the beginning, much more self-confidence. She was typical of many of our group mothers who come only because we insist on it. She saw no relation between her son's anti-social behavior and herself, for had she not always "beaten her brains out" to teach him right from wrong? She said the school principal "had a nerve" blaming her. Very soon she made the demand mothers of this kind always make for advice about handling her son. I said I knew that she and some of the others would probably be disappointed because I do not give advice. Experience had shown that it was best not to if we were to get to the bottom of the trouble. We found that the children's problems were usually related to the parents' emotional difficulties, so that the mothers would talk while I listened and together we would try to find the answers.

Mrs. P., a third member of our group, had so far sat by quietly, saying nothing. She was an untidy, withdrawn woman with a blinking tic. She was a college woman who, because of a deep sense of inadequacy, had married an uneducated man late in life. She was now at a complete loss as to how to cope with her two small boys. She was not really good group material but we had no other time for her and decided to risk it.

Now, for the first time, she took part in the discussion, quoting from

books (she was a librarian) to give Mrs. L. the advice I had withheld. I remarked that she seemed to agree with Mrs. L. in that I should give advice. She blushed and said "Well, no, but,—" I smiled in a friendly way and said that here everyone was entitled to her own opinion and that all mothers feel the same way at first. I realized I would have another set of intellectual defenses to cope with here and, furthermore, that Mrs. P. was the kind of patient with so little ego that I must go very slowly in tackling even her resistance. It is best to let such women sit quietly by until, from hearing the others express all sorts of emotions, they have gained sufficient reassurance to be able to touch on their own.

I shall omit the fourth woman from the discussion because she had least impact on Mrs. S. and it is necessary to save space.

Gradually, the women came to talk less and less about their children's behavior. (From then on, when they did revert to it, I would know that some new resistance was making itself felt.) They spoke more about their feelings toward them, their protectiveness, their feeling of responsibility and guilt and, in the end, their resentment and hostility toward them. Some groups are so emotionally mobile that they can even be helped to accept their death wishes in these first months of treatment. This group was far from ready for it. In some groups, where one or two members may go this far while the others would be shocked by it, the therapist must be especially careful to handle the feelings of both kinds of women.

The therapist's part in this group was to help them express these facets of their parent-child relationships freely, to show them the meaning of parental ambivalence and to help them see that they were not alone in their various predicaments.

During this period, Mrs. S. was the chief catalytic agent, leading the other women gradually to more personal problems because of her emphasis on her own childhood. Mrs. L. responded by telling of her childhood experiences, too. She criticized her mother freely. In her characteristic way, she showed her sibling rivalry by aggressively taking the floor most of the hour. She complained of being snubbed and ignored by so-called friends for whom she tried to do too much. Gradually, she began to see that she was not just the victim of their hostility but that she herself activated it by the chip on her shoulder. The same situation appeared to hold in her relationship with her son and, as a result, she began to treat him more tolerantly.

Mrs. P. talked freely at this time, too, for she half lived in her childhood fantasies anyway. Becoming aware of the meaning of some of them (her driving competition with her mother) seemed to bring her into somewhat closer touch with her daily life.

Since the others gave Mrs. S. little chance to talk these days, another of her defensive patterns was high-lighted. She appeared to be listening sympathetically. When she finally got the floor she complained of one of her severe headaches. She thought it was because someone had called

her as she was hurrying to get to our meeting. It was the head of a committee who told her a long story of woe in order to convince her that she should take over some onerous duties. Mrs. S. had listened with apparent sympathy, although she was angry. She took the job, but now felt overburdened and inadequate. I said I had an idea she was feeling the same way about the group lately when she sat for such long stretches listening with apparent interest to the other women's troubles. I knew this must be frustrating to her. Mrs. S. laughed a little and said she had also been having headaches after our meetings lately. I tried to get her to talk about these headaches, from which she had been suffering all her life. She started talking about them, but soon drifted off into a long story about how angry she became when her husband insisted on taking the whole family on endless drives and then expected dinner in a jiffy after she got home. As a matter of fact, she said, she often had headaches on Sunday. This demonstrated the feeling behind the headaches, and she laughed again as she put two and two together. Then she suddenly recalled an illuminating childhood memory. When she was about nine years old, she used to go to an art class. The other children used to make many demands on the teacher whereas she sat in a corner not daring to ask for help, although she could never draw as well as they. When she went home, she used to cry herself to sleep. I asked why she thought she had just recalled this. It was easy for her to see that she was doing something very similar here.

From that point on, Mrs. S. became a little more aggressive in the group. Soon there were several signs that her hostility toward her mother, of whom she had said only the most complimentary things so far, was mounting. She again became anxious and resistant, even though she had been somewhat prepared for the recognition of such feelings in herself by Mrs. L.'s open, bitter criticism of her mother.

When her resistance had been broken through, Mrs. S. revealed a negative transference to Mrs. L., whose overprotection of her son reminded her of her own mother's attitude. At this time, the group was talking about how to tell children the facts of life. Mrs. L. revealed that she could not talk about it to her son; that she worried about whether he masturbated; sometimes she even spied on him. Mrs. S. looking angry and upset, I said, "You seem to have some feelings about this, Mrs. S." In a cold, intellectual way, she said that a child's reaction to such handling could only be one of rebellion. I said, "I think you are feeling irritated at Mrs. L. for the way she treats Charles." She broke through then and said angrily, "Yes, he'll never forgive her, you know," to which I replied, "This seems to be the way you feel too; I think she reminds you of your mother." She burst out, "That's right, I'll never forgive my mother. She always pretended that sex was all romance and then one day she called us children together and told us the 'bestial side of sex' all at once. She made it sound so gruesome I never have got over it. It just about spoiled the first part of my marriage"—and as usual she cried.

From then on, her mother came in for one bout of criticism after another, until she reached a climax when she was telling us about her mother's irritating "sweetness and light" attitude when she came to take care of her grandchildren. Grandma always sidetracked them when they were going to be aggressive, and Mrs. S. felt that was at the bottom of her little girl's shyness and inability to be aggressive, just as it was at the bottom of her own inability to assert herself. Her voice rose in a crescendo as she burst out. "Pretending, pretending, that's all my life has ever amounted to—no wonder I can't manage my household, my children, or anything." She began to cry bitterly. While she was working through this hostility, her real relationship with her mother, who was visiting her, was very much disturbed. She had things out with her mother regularly. Her husband called me to ask what was going on, and I assured him that her reactions were an unavoidable part of treatment, but I felt they would soon subside and she would find a better equilibrium as a result. Fortunately, it was not long before Mrs. S. reported that she was much better able to manage her children now and that her daughter was much more aggressive than she ever had been. The incidents she told seemed to show that Mrs. S. was asserting herself in a more adult way at home. The explanation seemed to me to be that as soon as she was able to face aggressive feelings toward her mother, the need to remain a child in order to suppress her feelings of hostile rivalry toward her was greatly reduced. She could be the mother to her own children now instead of being just another guilty child with them. She was also getting on better with her mother-in-law, who no longer was the recipient of hostility meant for her mother. Her husband also apparently responded to her increased maturity, for she told us he had been so attentive she felt as if she were a bride again.

By that time, most of the women had talked a lot about their resentment toward the various members of their family, but they had not yet been able to criticize me directly. As in all matters of aggression, Mrs. L. was the leader. One morning, when they were all resistant at once, the conversation turned toward teachers. Mrs. L. (to whom I had recently had to deny information about what her son had told his therapist) said, "Teachers never tell you anything. When I want to know something, I ignore them and go straight to the principal," and Mrs. S. chipped in, "I think they ought to learn some psychology." (Note the appeal for my approval.) "They think they can handle all children the same way" (objection to group). Mrs. P. complained that, at school meetings, the teacher never seemed to notice her, so that she never had a chance to talk to her about her little boy.

Enough material about their familial backgrounds had come out by this time so that I could easily show them all that this was exactly how they had felt about their mothers, persons in authority, and, more recently, about me. Each one, in her characteristic pattern, had presented a picture of her own irrational idea of my attitude toward her. I showed

them each the meaning of what they had been saying. Insight was stimulated because each woman could see so clearly that what I said about the others was true, and they all laughed. Because the whole group was expressing resentment toward me at once here, it was possible for each to accept such feelings in herself at this time (security from the gang).

Following this episode, Mrs. S. expressed resentment to me more directly, but the persistent struggle of her need for approval over her emerging hostile feelings made treatment a slow, laborious process for her. The insight she gained was partial and frequently repressed again, as often happens. One episode threw into clear relief the way she used an appeal for approval as a means of defense against anxiety about hostile feelings. She was expressing resistance again one day, by talking about her dislike of doctors, when she stopped abruptly and asked me if fear could be repressed. I said, "Suppose you tell me what you mean." She told us about an operation she had undergone several years before. She had been calm beforehand and was often complimented by the day nurses. The night before the operation, however, she could not go to sleep, and one of the night nurses scolded her severely for it. Suddenly she became panicky and the next morning she insisted on calling up her doctor to ask him some questions. He told her abruptly that she was afraid. She referred to her calmness until this time. He answered harshly that she had only repressed her fears. She was angry, but helpless and more panicky than ever about the operation. She felt he had had no business making interpretations. As she talked about it I helped her see that she had been all right until someone had criticized her, as if being approved of (loved) had been a way of keeping down panic. I showed her, too, how the same pattern was working in the group. She had often tried to win my approval (this much had come out frequently and she had long since accepted it), and when I did not give it to her she became angry, panicky, and resistant.

It was clear now why Mrs. S. had come to treatment. Ostensibly, it was to learn how to handle her affairs. What she wanted, unconsciously, was to get approval from an authority so that she could continue to suppress hostility and deeper fears. It is clear, too, that had the therapist given her advice or praise he would have strengthened her defenses and made it harder, if not impossible, to work through the anxiety-laden drives that lay behind them.

When she was a child, her family's defensive generosity with money and praise had served to keep up her defenses, although they often wore thin and she had actually had a nervous breakdown at college. When she married, her husband and critical mother-in-law withheld both money and approval, and the defenses broke down. She was both angry and filled with a deep sense of inadequacy.*

When the same pattern had played itself out in treatment, the vicious

* Her basic fears had to do with castration anxiety which had frequently been indicated, but which she had certainly not been ready to touch so far.

circle could be broken because the therapist neither played into her defensive patterns nor rejected her, but showed her the meaning of what she was feeling.

What had kept her in treatment so long in spite of the enormous struggle she had to undergo to face her hostility, was a deeply passive dependent drive. I was her last hope of help and she had to stick to it. (Had the therapist been punishing or masochistic, she could not have stayed.) Her passive trends had also been evident from the very beginning and, by this time, she had gained some understanding of them. But she could not experience the tender and erotic nature of the feelings connected with the dependent drive until she was released by the expressions of resentment in a situation which did not bring with it the rejection or the retaliation she feared. I will not attempt to describe the slow steps by which she became aware of what she called "wanting to lean." The incident in which she recognized the connection between this drive and her hostile feelings, however, is worth recording because, without it, it is not possible to understand how she came at last to grapple with still deeper layers of anxiety.

Toward the end of our second year of work (about the 60th interview), when the others were complaining about their children again, Mrs. S. joined them, this time without any attempt to avoid my disapproval (heretofore, she had always worked in some compliment to me, for safety). She implied that her little girl was worse now than when she had first brought her to the center (in spite of the fact that she had already told us that the nail-biting and enuresis had stopped). I was glad to see that hostility to the therapist was stronger now than her need to side with the mother (myself) against the siblings as she used to do. Here we see a decrease in dependency.

She denied my interpretation of her doubts about treatment, but the next week she came in feeling depressed. She was worried, she said, lest she and her husband would have to help support her aunts who were now trying to live on their pensions. Using a deadly monotone, she gave the minutest details about how these aunts had helped her out financially, through college. Now she felt mean to begrudge them help.

I had only to remind her that this tone of voice usually meant resistance, and she immediately spoke in a more heartfelt way. The youngest of these aunts was very important to her because she had been the only bright spot in Mrs. S.'s life the year her sister had been sick. The rest of the family's attention had been concentrated entirely on the invalid, and only this aunt had paid any attention to Mrs. S. It was possible to show her how she was struggling here with guilty feelings about being hostile to someone she wanted to lean on. I showed her, too, how she was feeling just that way toward me at this time—needing my help and feeling guilty when she was angry or resistant so that she did not want to talk in the group. She indicated her growing insight by giving examples of how this same interplay of feelings had often happened in her

relationship with her mother, although she had never before known what it was all about.

The following week, Mrs. S. came in a warm friendly mood such as she had never shown before. She thanked me and said she was touched by my desire to help her. She spoke tenderly of her aunt who, she said, reminded her of her father: they both loved church music. Even now she gets a thrill whenever she hears organ music. Soon this was her theme for the hour. It was the first time she had said much about her relationship with her father, and the first time that genuine tender, erotically tinged feeling came out. Aunt, father, therapist seemed to be equated here.

Apparently, it had been even harder for Mrs. S. to express affection, which was choked off by such angry feelings that she feared rebuff and even retaliation. The praise her mother had offered her had merely covered up basic rejection and preference for Mrs. S.'s brother. Our patient had, therefore, found herself in constant need for an expression of love, or its substitute—approval. Never satisfied, she kept asking for more, repressing the hostility which she felt might cut her off altogether. Now, at last, she could express love and she looked much happier as a result.

At the last visit before vacation, Mrs. S. expressed regret at leaving, but compared herself favorably to the year before, when she had been terrified of the summer vacation. She had an excellent summer and when she returned in the fall she seemed an entirely different patient, for she plunged with real feeling and spontaneity into some of her underlying conflicts. It was apparent that we were through the worst resistance. She tackled first her difficult, partially frigid sexual relationship with her husband, which heretofore she had declared was one thing with which she needed no help. Next, she came face to face with her inability to assume either a feminine or a masculine role (which was the real significance of her intellectual strivings). Trying to compete with men made her feel hypocritical and "hollow." She always felt she might be found out at any moment. On the other hand, being a woman always made her feel dirty and castrated. Working out some of the hidden feelings and fantasies surrounding this basic conflict gave her enormous relief, making it possible for her to express franker sexual feelings toward her husband. She has been looking radiant of late and speaks of experiencing a new "*joie de vivre*" entirely unknown to her. Her housekeeping is improving at last, and the family manages to have more fun when they are together. It seems clear that, by the end of the year, she will have worked out a much better relationship with her husband, for he seems to be responding to her own changed attitudes.

In spite of the fact that I have selected only a few of the highlights of a long, tedious process, it seems clear that treatment is not a series of beautiful insights. It is more like putting together the tiny parts of a jig-saw puzzle. The fact that there are three other women to be dealt with makes the puzzle more complex and one is frequently distracted

from one by the others, but, on the other hand, they offer many clues and stimulation that would never occur in a two-way relationship. This therapy offers even the beginner a safe guide through what often seems a dense fog. If he concentrates on sensing and handling only the immediately underlying feelings that the patients are experiencing at any given time, he will succeed in gradually building up a clear picture and helping his patients to richer personalities and fuller lives.

PSYCHODRAMA AND GROUP PSYCHOTHERAPY

By J. L. MORENO

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IN the last two centuries, three revolutions have taken place in the field of psychotherapy. Each was characterized by a specific change of operation. In each case, the new practice gradually compelled an overhauling of theory, but in each successive case the new method was broader in scope and, to a degree, included the previous form.

The first, in the middle of the eighteenth century, is connected with the name of a Viennese physician, Mesmer. The operation, *hypnosis*, consisted in putting the patient into a state of trance. Mesmer thought that the hypnotist is responsible for the state of the "hypnotizand" and developed a theory about animal magnetism according to which a fluid travels from the physician to the subject.

At the end of the nineteenth century, another Viennese, Freud, brought about a new revolution by discarding the hypnotic sleep as a means of treatment and establishing another form of operation. Patient and physician faced each other in full consciousness, the patient was told to tell the doctor whatever came to his mind. The physician expected to attain by this method, which he called *psychoanalysis*, all the results which had been attained previously by means of hypnosis and many more things to which the hypnotized state of the patient closed the doors. The psychoanalytic method of operation brought Mesmer's theory of animal magnetism and all its intellectual modifications *via* Charcot, Bernheim, and others into discard, and it was replaced by the well-known system of psychoanalytic theories.

During the crucial years between 1900 and 1925 in which psychoanalytic theory and practice developed, there have been many widely discussed differences between psychoanalytic schools. However, the conflict between Freud, Jung and Adler was due to different views of analysis and interpretation, and there was no conflict between them as to *operation*. The Freudians emphasized libido and its cathexis as chief determinant of human behavior, while Adler preferred inferiority organs and inferiority feelings as the core of his analysis, and for Jung it was the collective unconscious and the extrovert-introvert types of personality which seemed to matter. But if we could have entered the office of a Freudian, an Adlerian or a Jungian between 1910-1930, the operation would have been about the same: a physician and a patient alone, in a doctor's office strictly private and sealed from observers. There were slight modifications: in one case a patient relaxing on a couch, in another case facing him, the patient sitting in front of the doctor, in still another case the procedure being more informal and the duration of the

treatment shorter. But in all cases the patient would have been found talking freely about himself and the physician giving an analysis of the material elicited. However great the contrast may have been in the ways of interpretation and in its depths, there was no difference in operation.

In our own time, in the last twenty-five years, a new revolution took place when the first therapeutic theatre was started in Vienna. It was again due to a radical change of operation. The method has become known as psychodrama, sociodrama, role playing, and action taking. *The patient is now an actor on the stage, acting before a smaller or larger audience of other patients.* The physician-patient relation has become subsidiary. Again, we are in the midst of an overhauling of theory. With the new operation, new concepts and theories are emerging. It consists of two procedures: (a) treatment of the audience (group psychotherapy); (b) representatives of the group portray on the stage the problem from which the audience ails (action therapy). The group is facing the mirror of itself (in many versions) on the stage. It looks into this mirror and sees itself. The responses coming from the shock to the audio egos (members of the audience) and to the auxiliary egos (actors on the stage) are systematically followed up.

DISCUSSION ON GROUP THERAPY

DR. S. R. SLAVSON (*New York, N. Y.*):

DR. DURKIN's paper* demonstrates the very significant fact that group psychotherapy is not different in any essential respects from individual treatment. We have seen in her paper how the focus of therapy is the individual patient and that the group is used only as a tool in treatment, rather than being its center.

Group psychotherapy must be considered as only one form of psychological therapy and is an integral part of it. It bases itself upon the same understandings and employs precisely the same dynamics as does individual therapy. However, the presence of more than one patient and the interstimulation that occurs as a result of this, aid or retard the therapeutic process for each in accordance with the situation. As individual psychotherapy, group psychotherapy is also based upon five dynamics: (1) relationship, (2) catharsis, (3) insight or ego-strengthening, (4) reality testing, and (5) sublimation.

Relationship and transference are too well accepted and understood to need elaboration here. Catharsis as it appears in group therapy, however, is somewhat different. Verbal catharsis is employed in interview group therapy, while activity catharsis prevails in activity groups. Children of pre-school and school age can express their feelings and attitudes through action more freely and appropriately than through discussion. While insight in group psychotherapy is not as profound and deep as in psychoanalysis, the aim is to help each patient in the group to gain some understanding of his motivations and behavior in terms of unconscious conflicts and strivings, as well as in the light of his emotional history. This latter fact has been particularly well demonstrated in Dr. Durkin's paper.

In our own work with activity groups, we found that even in such groups where no discussions are held or interpretations given, children become aware of their own changes in attitude and alterations in feeling tones. Frequently they verbalize this, although most often it remains unformulated in words. This understanding is evidenced in many ways even when it is not verbalized, and has been described as *derivative insight*.

Reality testing is the fourth dynamic operative in all psychotherapy. In fact, in some respects, this is the greatest contribution of group therapy to the therapeutic process. Each individual tests himself against reality in everyday pursuits and contacts, and patients meet the pressures of the milieu in various ways. They observe how adequately they deal with reality and how much they are hurt by it. In accordance with his basic problem, the patient may become latently hostile, overtly aggressive, or he may withdraw. As he finds himself adequate or wanting,

* See pages 389-901 of this monograph.

he returns to the therapeutic situation to examine himself or gain support from the therapist. To give this support is an important function of the therapist, whether in individual or group treatment.

The group is a more tangible reality than is the individual interview. Here are persons who also have problems, and the best results are obtained where the psychologic syndrome (though not necessarily the symptom) is similar in all the members of the group. There are present hostilities, rivalries, jealousies, antagonistic and ego cravings, mutualistic support. The patient finds himself in a realistic situation and can test himself within the therapeutic situation itself. Thus, therapy and reality are fused into one. Although this unitary relation is not essential to obtain results in therapy, it accelerates the process greatly. There are patients to whom this confluence of therapy and reality is helpful, if not essential. The very important point to be borne in mind, however, is that group therapy is in essential respects the same as individual psychotherapy and that it is based upon the same concepts and dynamics.

In group therapy, transference (relationship) is greatly modified through the network of emotions in the group. In addition to the attitudes toward the therapist, there also exist sibling relations and various identifications. Thus, the transference is *diluted*. Catharsis emanates not only from the transference relation but it is also stimulated by the other patients, and by the fact that anxiety is less intense in a group.

One point needs to be stressed and this is the factor of *ego-strengthening*. While ego-strengthening does occur in all therapy, it is the very foundation of activity groups. The child brings his impulses under control through the pressures of the group and through the fact that he gains status and acceptance. To the child, whose character is not fully formed and set, experience takes the place of insight. We found that insight for the young child is not as important as are release and strengthening of his ego.

Another dynamic that helps the therapeutic process in a group is *target multiplicity*. The therapist is not the only one who receives the hostility and love of the patients. Feelings are displaced on or redirected to other members of the group. In activity groups, the child destroys objects in the room or he attacks other children to displace hostility toward the therapist. In interview groups, destructive attitudes and emotions are redirected toward other members of the group.*

Another important dynamic that is present in therapy groups as described by Dr. Durkin can be termed as *cathexis displacement*. This is the dynamic in which emotional ties are established with the therapist, other members of the group, or the group as a whole. The outcome of this process is that the patient becomes more emotionally free as his earlier ties are disengaged from their infantile anchorings.

Since each patient feels that his problem is not peculiar to himself and

* See SLAVSON, S. R. Differential dynamics of interview and activity group therapy. *Am. J. Orthopsychiat.* April, 1947.

that others are in the same situation as he, his deflated self-evaluation is repaired and his ego strengthened as a result. He is thus able to deal with himself and the world around him more effectively. This we have described as the process of *universalization*.

Dr. Moreno's contribution to the field of psychodrama need not be re-emphasized here.* Its value for specific patients has been amply demonstrated and many of his formulations have now been incorporated in the general psychiatric literature. It will be of great interest to examine further the nature of the relationships in the psychodramatic situation in terms of group dynamics, which I hope will be undertaken in the near future. This should prove a very fruitful field.

* Cf. pages 902-903 of this monograph.

WHAT DID THE CLINICAL PSYCHOLOGIST LEARN FROM THE WAR?

By MAX L. HUTT

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IF we attempt to answer the question contained in the title in terms of the evolution of entirely new techniques of measurement or in terms of startlingly new concepts of personality or mental disease, we shall find that our search for such developments is a vain one, for, by and large, so far as the writer knows, no great discoveries in clinical psychology were uncovered during the war. Nevertheless, the many psychologists from all of the branches of military service with whom the writer has talked, and the many clinical psychologists with whom he was privileged to work in the army, would all agree that a great deal was learned. Nor would they be content with an assertion that the result of this learning process was indefinable, for there were many specific and concrete gains. The difficulty in specifying these gains is that some of them were gains in a negative sense, and another is that the gains were not always in terms of tests and devices but rather in techniques and approaches. In fact, one can sum up our war experience with two complementary, although *apparently* contradictory statements. The first is the humbling conclusion that the scope of our ignorance in the field of clinical psychology is still appallingly vast. The other is that, as the result of the pressing needs of the military situation and of an unprecedented array of clinical data, many important, if not epochal, advances were made in technique and some in theory.

If our previous training and experience did not yield completely satisfactory answers to the needs of the war situation, they did at least enable us to get adapted to these needs in many ingenious and practical ways. We shall attempt to discuss these advances made during the war under four major headings.

Extension of Basic Clinical Experience. The experience of psychologists who worked in the Medical Corps, and especially in the Neuropsychiatric Division, was broadened and deepened in at least three ways. The most obvious of these was in the extension of clinical practice to a large number and a great variety of neuropsychiatric and special medical cases. These cases differed from those usually seen in state institutions or in community clinics. They were essentially non-institutional cases, young adults representing a cross-section of the healthiest segment of our population. There were relatively few chronic neurotics and deteriorated psychotics but there were many acute neuroses and neurotic reactions and many acute or incipient psychoses. In civilian life, we rarely

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had the opportunity of examining or treating such people. They were in the borderland of psychiatry or even beyond these borders and rarely came to the clinic or hospital. In addition, however, there were many supposedly non-neuropsychiatric cases, the many general medical cases who manifested psychosomatic disturbances or were "cured" medically but did not get well. There were also hundreds of cases of diagnosed aphasia, hundreds of paraplegics and thousands of amputees, blinded, deafened and the like, as well as large numbers of cases with recent brain damage.

Clinical psychologists had worked with few such cases, from the young adult population, prior to the war, and they soon learned that the previously used tests, differential signs and clinical symptoms could not be applied directly to these individuals. Moreover, they were confronted with individuals coming from all parts of the country, with extreme variations in education, social experience, and occupational background. These background variations may have been known to psychologists from their clinical literature, but few of them had ever been confronted by such variations in their home communities.

A second aspect of the extension of clinical experience was in the field of guidance and therapy. Although in many cases psychologists were unprepared or only partially prepared to offer therapy, the tremendous number of cases requiring therapeutic care compelled the use of clinical psychologists in therapy. In a survey made by the writer during the last year of the war, it was found that clinical psychologists assigned to army medical installations were devoting, on the average, 25 per cent of their total duty hours to psychotherapy. Their roles in therapy varied largely in accordance with their training and interests and, to a considerable extent, with the attitudes of the psychiatrists to whom they were responsible. Most therapeutic work was on an individual short-term basis, averaging 5 to 10 sessions per patient. The second largest type of therapeutic aid given was in group therapy, which was explored more fully in the war than had ever been the case previously. Some psychologists directed comprehensive rehabilitation programs involving the use of fairly large numbers of ancillary workers.

Such diversified therapeutic programs offered psychologists opportunities they were often denied in civilian practice. They had to learn while they worked, and learn they did in more ways than one. It is not possible to discuss in this report the varied types of therapy employed, because of lack of space. Suffice it to say that, despite the extensive experience many psychologists had had in the Army, most felt that they could profit from further systematic study of this field, supplemented by appropriate extensive clinical experience. As an aside, it may be indicated that group therapy, and by this I do not mean simply group mental hygiene discussions, was found to be a much more worth-while procedure than many had anticipated, and in some cases group therapy became the therapy of choice.

A third type of extension of clinical experience was found in working as a member of a neuropsychiatric team. To many psychologists, the team concept was a new one. By army directive, psychologists became members of a team consisting of at least a psychiatrist, a psychologist, and a psychiatric social worker. These members had to learn to work together, to supplement each other, and to accept designated responsibilities.

There was, and still is, considerable difference of opinion, and even confusion, about the team concept. In some cases, personal and professional jealousies jeopardized the functioning of the team and did not bode well for the patient. On the whole, however, the concept of the neuropsychiatric team, which did not arise in the war but was certainly strengthened during it, was accepted, and the teams functioned harmoniously. Partly as a result of the war experience, the main problem today seems not to be whether to accept the team concept in neuropsychiatric practice, but rather defining and analyzing the concept more adequately.

The extensions of clinical experience which we have discussed suggest important implications for training and research, which are not, however, within the scope of this paper.

Psychometric Problems. No attention will be given in this report to the numerous screening tests and the special trade and aptitude tests and other assessment procedures developed by both the Army and Navy. Noteworthy as these methods were with respect to theory of testing and methods of test construction, they were not essentially clinical instruments. We shall confine our inspection to individual clinical devices.

In the Army, according to a survey made by the writer, clinical psychologists devoted about 30-35 per cent of their time to individual testing of intelligence, personality, special disabilities, and the like. This was their most important function in terms of both time and significance. However, the psychologist was no longer a mere psychometrist. He was essentially a psychodiagnostician. Numerical scores on tests assumed their rightful place in clinical work as minor aspects of the results of such testing. On the whole, the very marked emphasis was upon understanding the psychodynamics of the patient. To this end, both objective and projective tests contributed significantly. In neither case, however, was the calculation of a score, the construction of a scattergram, or the summation of the quantitative aspects of the test protocol an end in itself. These became the bases upon which cues about the personality or hypotheses about the patient's difficulty could be developed. Some cases were referred, of course, for determination of possible mental deficiency, where some quantitative results were particularly important. Even here, however, much more was usually demanded.

Psychologists made increasing use of observations of the subject during the test situation and made qualitative analyses of test responses.

In a sense, this was forced upon the psychologist, not only by the insistent requests for differential diagnosis, dynamisms of adjustment, and leads for therapy, but equally as much by the plain fact that the standard test profiles, scattergrams and signs *did not fit* the cases being referred for evaluation. The nature of the problems encountered and the cases referred has already been noted in the previous section of this report. These problems were unique. Old normative data were often inadequate. Hence, the more expert psychologists relied more and more upon truly clinical evaluation of test and observational data. When this was not done, serious errors in evaluation often resulted.

Hence, it followed that intelligence tests, while used very frequently, were more often analyzed for evaluation of the personality than for a rating of intelligence. The Army Wechsler Test and later the Wechsler-Bellevue Scale was the test of choice because it lent itself to such evaluations. As already noted, scatter analysis went an important step beyond simple test ratings, but it did not go far enough. The intelligence test was analyzed for content of responses and for clinical behavior elicited. Dr. Hunt has stressed the importance of this type of evidence in his recent paper in the *Journal of Clinical Psychology*.^{*} The writer wishes to confirm Hunt's conclusion that there is far too little published material concerning the qualitative data yielded by the individual intelligence test. This lack becomes increasingly important in view of the developing function of the neuropsychiatric team and the increasing emphasis placed upon the diagnostic skill of the psychologist.

Projective tests of many kinds were used extensively during the war and were relied upon more heavily than any other type of test for intensive personality evaluation. It is the writer's opinion that this type of test will be used much more extensively as a result of the war experience and that a great deal of research with many kinds of projective devices will be done in the near future. The possibilities of analyzing not only personality structure, but also specific complexes, conflicts, ideations and attitudes are indeed tremendous. We are only on the threshold regarding our use of this method. While much of the data yielded by such tests can be obtained in intensive psychotherapeutic work, these data can often be secured more quickly and more completely by projective analysis.

With respect to the problems of aphasia and mental deterioration, our military experience has convinced many that the psychological tests for these conditions are hardly adequate. On the basis of this experience, several studies have already been undertaken to gain a better understanding and to develop more suitable tests than were heretofore available.

Clinical Procedures. We have already alluded to changes in clinical procedures in previous sections of this report. Only one aspect of this

^{*} See HUNT, W. A. 1946. The future of diagnostic testing in clinical psychology. *J. Clin. Psychol.* 2: 311-317.

problem will be discussed here. There appears to be a fairly sharp division of opinion concerning the organization of test schedules or batteries of tests to meet the needs of clinical work. One group suggests the routine use of a comprehensive battery of tests for all or most patients. This battery is supplemented occasionally by special tests in exceptional cases. Some of the arguments in favor of such a procedure are: (1) comparable data on a number of tests become available for research; (2) a routine battery of tests offers a systematic check on the most important phases of the patient's personality; and (3) the psychologist gains familiarity with the battery and is more readily able to integrate the findings successfully. These advantages are real and important. However, the war experience has convinced the writer that such a procedure generally is wasteful of both the patient's and the examiner's time and that it does not provide for the more detailed probing required in psychiatric illness. This experience suggests the advisability of tailoring the battery to the individual requirements of the case. Different kinds of cases require markedly different batteries of tests. It is a gross waste of time to test routinely with the entire battery for all cases. Moreover, special attention can be given to those aspects of the problem needing further analysis. In some cases, indeed, only one or even no tests at all need be given. If equal amounts of time are expended with each method, the latter approach, requiring careful prior analysis of the needs of the particular patient, will probably reveal more important data for the time allotted, since only the area about which some question is raised will be explored. There is also the danger in the routine use of a battery that the approach will become mechanical and significant clues neglected. The flexible battery invites the development of sensitivity to the needs of the case and of adaptations of tests and test batteries to fit those needs. In any case, whether one accepts the first or the second view, it is clear from our experience during the war that we can expect the development of new types of test batteries for varied clinical use.

Clinical Theory and Nosology. Relatively little that is new was learned concerning the theory of psychiatric illness. In the main, the contribution of the war experience was to highlight certain conceptions and to afford an opportunity of testing some therapeutic approaches, *i.e.*, shock therapy, narco-synthesis, hypnoanalysis, and the like. Although they had been known previously, the war sharpened some concepts considerably. For example, the distinction between "traumatic reactions" and chronic neuroses became much clearer and the distinctions between "acute psychotic episodes" and "essential psychoses" became more evident. The effects of recent traumatic brain damage were studied intensively and on a wide scale. The psychologist contributed to the charting of these distinctions, and much of the exploratory work remains to be published. It was as if a great experiment in evaluating the effects of sudden and of prolonged trauma upon personality structure and func-

tion was being conducted. Conventional psychiatric labels were often wholly inappropriate, so that new nosological classifications had to be and were developed. The role of certain etiological factors in breakdown, especially the breakdown of the so-called "normal adult," was studied, if not systematically, at least extensively. As classification of mental illness improved, diagnostic techniques also tended to improve. Some of these advances have already been reported fully in psychological and psychiatric literature in the past 2-3 years and need not be repeated here.

Summary

The impact of the war upon clinical psychology has been great, although no startlingly new developments took place. The extension of the boundaries of clinical psychology, the deepening of experience with tests as diagnostic instruments and with psychotherapy, the adaptation of clinical techniques to meet diverse problems of new types, and the improvement in our knowledge concerning the theory of mental illness are all resultants of this impact. Inadequacies in our present clinical knowledge and, in particular, in our training programs have been emphasized. One thing more needs to be added: The profession of clinical psychology, as a professional group, was ill prepared to meet the needs and the opportunities created by the war. Clinical psychology was not represented at the highest levels of military command until late in the war, and even then not adequately. However, the war has served as a great catalytic agent in the development of clinical psychology, and its effects will be evident for many years to come.

FUTURE TRAINING IN CLINICAL PSYCHOLOGY

By JAMES G. MILLER

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THE tremendous increase in demand for the services of professional personnel qualified to take part in the care of neuropsychiatric illnesses results from an increased ability, on the part of both physicians and laity, to recognize the presence of these disabilities; from a greater willingness of the general public to admit that they are suffering from such disturbances—a consequence of the general educational campaign which has caused them to be viewed more understandingly; and also perhaps from changes in the cultural patterns of this modern age which may serve to raise the incidence of such illnesses. Whatever the causes for this demand may be, it is clear that the medical profession cannot adequately meet it alone. The services of clinical psychologists, psychiatric social workers, and related professions will be needed in very large numbers, besides the skills of neuropsychiatrists.

Before the war, clinical psychologists were employed sporadically in private hospitals, clinics, and guidance centers, as well as in state hospitals and clinics. Only a handful were employed by the federal government. In private and state institutions, the demand for clinical psychologists is growing rapidly, but the most dramatic outgrowth of the war has been the expansion of large government programs in this field, particularly in the United States Public Health Service and the Veterans Administration. Eventually, the program of the Public Health Service in its central and decentralized aspects may well be larger than that of the Veterans Administration, since it has responsibility for the mental health of seven-eighths of our population. The Veterans Administration has responsibility for only one-eighth, but its rapid expansion immediately at the end of the war has enabled it to get under way earlier.

It is expected that clinical psychologists in the Veterans Administration will be employed in at least five sorts of installations—general medical and surgical hospitals, neuropsychiatric hospitals, mental hygiene clinics, aphasia centers, and paraplegia centers—the latter two located in general medical and surgical hospitals. In these installations, they will work in the neuropsychiatric team, sharing a large part of the responsibility for diagnosis, research and therapy in connection with the psychiatric and psychological care of the veteran. Intensive efforts have been made by the Veterans Administration to recruit clinical psychologists, but these were doomed to poor success since the organization could effectively employ at least twice as many qualified clinical psychologists as there are in the whole country. The only solution to the great shortage in this field was obviously the establishment of a training program.

The Veterans Administration has therefore determined upon the policy of sponsoring a large-scale training program leading to the doctoral degree in clinical psychology in collaboration with those universities accredited by the American Psychological Association in this field. In 1946-47, 22 such universities cooperated with the Veterans Administration, taking 218 trainees. In 1947-48 the number of universities is 36 and the number of trainees 468.

Duties for which the Neuropsychiatric Team must be Trained. The training in psychological fields made necessary by the clinical programs now developing may reasonably be discussed at two levels: first, what would be desired if circumstances were entirely favorable; and second, what can be accomplished out of the realities which prevail at present. In all likelihood, it will be possible gradually to improve the existing situation so that it approaches the ideal more nearly.

Let us first consider what would be the most satisfactory of all possible training centers in the field of professional psychological studies. At first, it would seem to be where every student receives full training in at least psychiatry, psychology, and psychiatric social work, three of the fields of application of the psychological sciences necessary to carry out the clinical responsibilities of any large neuropsychiatric program. Individuals receiving this inclusive education would be qualified to perform all, or nearly all, the duties involved in caring for psychiatric patients. This is a long list of functions, including arranging for intake, determining the chief complaint, obtaining the present history, taking a physical and psychiatric anamnesis and review by systems, getting corroborative history and other facts from the patient's family and friends or from institutions with which he has had contact; performing physical and neurological examinations; determining the mental status; requesting or performing indicated medical laboratory tests; carrying out necessary psychological examinations; making a diagnosis and prognosis; conducting therapy; directing occupational, recreational, physical, and other adjunctive therapies; doing research directed toward improving available clinical techniques; arranging for disposition of the patient; doing case work with his family and friends as required; and following the patient's later course for purposes of further treatment, maintaining records, and research.

The fact is, however, that it is not feasible for all this to be done by one individual. Even if one person could perform all these tasks at the highest level of professional accomplishment which has been reached—and no one could—it would still be true that division of labor is more efficient, for it has often been shown that a professional team can deal with a larger number of patients than they could if each person worked independently. Specialization has become a recognized necessity throughout the medical sciences, and the complexity of the clinical services which should be offered in the psychological field indicates clearly that

there must be specialization here also. For the most satisfactory care of mental patients, the adoption of the neuropsychiatric team, made up of a minimum of three professions—psychiatrists, clinical psychologists, and psychiatric social workers—is inescapable.

Another obvious fact leading to the same conclusion is that there are few individuals of sufficient motivation, economic security, or emotional stability to complete the years of study necessary for such inclusive training. Accomplishing complete education in all these fields is a utopian goal which can be met, at best, by very few.

Let us include specialization in our ideal professional situation, therefore, and attempt to determine the most satisfactory allocation of tasks to each of the three specialist groups. In doing this, we shall neglect such crass human considerations as restrictions of licensing, professional jealousies, and resistance to change, assuming naively that the sole concern of all is the welfare of the patients.

An Effort to Distribute the Duties Ideally. One distribution of responsibility for which precedent could be found would be for the psychiatric social worker to perform necessary intake tasks, make arrangements for disposition of the patient at the end of treatment, and see that he is available for follow-up, leaving the psychiatrists and clinical psychologists to divide responsibility for the other care of the patient. Conceivably, this arrangement might be satisfactory, but it is evident that such a division of labor creates problems.

For one thing, the patient has family and friends and is a component of society. There are reciprocal relations between the patient and those who constitute his environment, and whenever there is mental maladjustment in one person there must also be some maladjustment in those he contacts. If the husband is ill, the wife is affected; if the child has symptoms, they may be the symptoms of his mother's illness. Frequently, arbitrary considerations determine which member of the involved group is considered to be the patient and is treated by the psychiatrist. It may be the one who has the most overt symptoms; the one most willing to consult a doctor; or the one who came to the hospital for a medical condition and was referred to a psychiatrist. It may be that only the veteran in a maladjusted family can be treated, because only a veterans' clinic is nearby; or it may be that only the child can be seen, at a neighboring children's clinic. Traditionally, social workers do "case work," which is often a kind of therapy, with the non-patient members of the family. Since the selection of the patient is so arbitrary, however, no difference should ideally be permitted between the training of persons who treat non-patient members of the family and those who treat the patient. The fact that a social worker has the psychiatrist to turn to when she encounters difficulties may mitigate this inequality, but there is clear injustice unless all members of a maladjusted family group are cared for by individuals with the same sort of training, whatever that may be.

In making disposition of patients, one of the important tasks of social workers in the Veterans Administration and elsewhere is finding them jobs. In an ideal treatment center, persons doing this would have to be conversant with the whole range of possible vocations, know job descriptions accurately, and be able to administer and interpret vocational tests. Social workers are not at present trained in this field and, therefore, in ideal situations vocational psychologists would have to be employed for this part of disposition. For these few reasons, out of many which could be mentioned, we can see that the social worker's job cannot easily be categorized as "intake and disposition."

Recognizing that this is not a satisfactory definition of the scope of the social worker, let us, nevertheless, proceed to consider a possible distribution of the remaining duties, again dealing with a division of labor which can find plenty of precedent. Suppose that, in our clinic, the clinical psychologist does psychological testing and the psychiatrist carries out all other aspects of clinical care, from taking the chief complaint to returning the patient to the social worker for final disposition.

Such an arrangement might be workable if there were not more to psychological diagnostics than administering tests and adding up the scores. Much clinical skill is required in reporting even a simple I.Q. to make allowances for the patient's linguistic handicaps, the extensiveness of his education, his eyesight, his socio-economic background, his physical health, his *rappor*t with the tester, and many other considerations. Interpreting the Rorschach test or other projective procedures is much more complex. Relating objective diagnostic findings to the circumstances of the individual case can be done only if the interpreter has had enough clinical experience to understand thoroughly the problems of human personality and mental disease. Interpretation of psychological diagnostic procedures can best be done by the one who has administered them, but if the psychologist has done only routine testing he will be unable to make the most of his procedures.

If the psychologist is to be an ancillary technician in the complexities of psychiatric diagnosis, it must be in the way that the radiologist, rather than the laboratory biochemist, is a technician. In general medicine, it is clearly understood that the man who interprets x-rays must have a broad background including the whole range of clinical experience. The same is true of the psychological diagnostician of the future—the nuances of these diagnostic problems are too many and complex to be learned in the fastnesses apart from patients which can more reasonably be occupied by biochemical technicians. The psychological diagnostician who does not constantly submit his findings to the validation of the therapeutic course rapidly becomes removed from clinical reality, sterile, and esoteric.

Another consideration which relates to the advisability of limiting the role of clinical psychologists to testing and delegating nearly all other responsibilities to psychiatrists is that most training for psychiatrists

offers little opportunity for them to become conversant with psychological principles. They may take a course or two in psychology in college and one of the very feeble courses in this subject which some medical schools offer, but they are not thoroughly grounded in the wide field of experimentation and investigation which, at present, forms the systematic science of psychology. The average well-trained clinical psychologist knows more about the "physiology of the mind," the normal functioning of the personality, and related topics than does the average well-trained psychiatrist. At present, psychologists can contribute a great deal to interpretation of all sorts of behavior from such a background, and if the psychiatrists do not have this knowledge available to them, either from their own intensive study or from the comments of colleagues in the neuropsychiatric team, their handling of patients will be less effective. The systematic body of facts collected by investigation in the fields of experimental, physiological, comparative, child, social, and personological psychology are capable of innumerable sorts of clinical application. A beginning has scarcely been made in employing in the clinic the findings of the laboratory on such matters as maturation, perception, learning, memory, motivation, group behavior, and many others.

Because the region of the mental sciences is so little explored, emphasis on research should be of paramount importance in every psychiatric center. Much diagnostic and therapeutic work in psychiatry is so feeble that tremendous investigative efforts should be made to catch up with the other divisions of medical science. In most of these other divisions, there are many M.D.'s or Ph.D.'s trained in laboratory and clinical investigative science who devote a large part of their energies to advancing the field. Some of these not only understand experimental procedures but also have had clinical experience, which makes them more effective. The vast majority of psychiatrists lack the necessary training for research, and, because experimental institutes and laboratories in psychiatry are almost unheard of, it is understandable that their training has not motivated them for it. Many psychologists—by no means all—on the other hand, because of their professional philosophy and traditions, are intensely eager to do research. They are acquainted, by experience, with a number of methods applicable to clinical investigation concerning which most psychiatrists are ignorant. Unfortunately, they usually have not tried to get, or have not been permitted to obtain, sufficient clinical experience to enable them to apply this knowledge effectively. In their ignorance, they are likely to be supercilious and unreasonable in the demands for precision which they make of clinical research. Unless they take an integral part in clinical operations of all sorts, they will not be able to get the experience which will enable them to make the most satisfactory applications of their method and to fit it into normal clinical routine so that it will be beneficial rather than harmful to individual patients. An alternative is to make psychologists out of psychi-

atrists, so that they will be equipped with this technical background so valuable for research.

The conclusion to be drawn from this discussion of the ideal arrangement for rendering psychiatric services is that some degree of specialization is desirable from the point of view of efficiency and thoroughness of operation. However, it is not possible or desirable, even in an ideal set-up, to delimit sharply the activities of social worker, clinical psychologist, and psychiatrist. It is important for them to have certain types of overlapping functions. Such overlap must be recognized in ideal job descriptions for the three professions. When we can approximate such descriptions, we may proceed to consider what education each group should have in order to fulfil its ideal role. Finally, we may try to discover how closely it is possible, at present, to attain such ideal training.

Differences in the Backgrounds of the Professions. Let us deviate, at this point, to observe the present differences in background among the three professions. First, there are striking differences in education. Psychiatric social workers complete two years of graduate work to a master's degree. Clinical psychologists now usually complete three or four years of graduate education to a doctor's degree. Psychiatrists complete four graduate years for the M.D. and then have one to six years of further training, largely clinical. This is a wide discrepancy in the amount of preparation.

Characteristically, schools of social work base their curricula on the assumption of undergraduate training in related fields. This has the effect of lengthening professional training by beginning it before the graduate years. Most of these schools prefer or require for matriculation some systematic sequence of courses in the social and biological sciences, including sociology, anthropology, psychology, history, government, economics, and biology. The graduate work founded on this includes courses, reading, and field exercises designed to give training in the following areas: psychology, usually so taught as to emphasize the dynamics of personality mechanisms, and commonly not presented at an advanced level or by highly qualified psychologists; public welfare and administration; community organization; social agencies and institutions, public and private; social statistics, including extremely elementary instruction in research techniques and in the sorts of procedures necessary for compiling socio-economic data into tables and charts; elementary medicine, introductory psychiatry, and basic legal concepts relevant to social work; and economics.

The most significant part of the preparation of psychiatric social workers, however, is not courses but carefully supervised practical training in psychiatric case work. This involves detailed analysis of interviews, in many ways like the apprentice training of some psychiatrists, as well as practical direction as to how the many resources at the command of the social worker can be most effectively employed for the wel-

fare of the patient. Through an entire year of supervised internship, the student, under direction, learns by his own mistakes. Throughout all of this, a dynamic approach to human personality is adhered to, which is more commonly than not doctrinaire, being cast into the Freudian, the Rankian, or some other theoretical system. While the graduate training of social workers is officially two years in length, actually it has been traditional in the profession to continue this apprenticeship into the early years of full-time employment. This on-the-job training directed by case supervisors, even if not of formal character, is nevertheless an important educational experience, and it is important to realize that the two graduate years in social work are by no means the end of the preparation of a conscientious worker.

A final item in the education of a social worker often has been a psychoanalysis. Some schools have strongly opposed such training, while others have exerted such pressures in favor of it that their students have felt it to be essential, although there has not been any official requirement. Commonly, such pressure has been for personal rather than didactic analyses, and most of the analyses of social workers have not been of the training type, though of course they could not help but have educational importance. Though there has been, in recent years, perhaps more general acceptance of the value of psychoanalyses, it is probable that a smaller percentage of social workers are receiving them now than were ten years ago, because they are at present so difficult to obtain.

The training of clinical psychologists involves a markedly different subject-matter. Specific course work usually begins in undergraduate college and continues into graduate school. It includes beginning, abnormal, and experimental psychology; psychology of learning, perception, motivation, and the higher mental processes; theoretical systems in the psychology of personality and clinical psychology; mental hygiene; the administration of objective tests of intelligence, attitudes, aptitudes, and other traits; the use of projective techniques and other diagnostic procedures including mental status examinations; theory of interviewing and psychotherapy; statistics; theory of test construction, experimental design, and scientific method. Practical experience is given in the construction of educational, vocational, industrial and clinical testing procedures; in the use of all these methods; and in the conducting of independent research. Supervised internships of various lengths usually involve the application of diagnostic rather than therapeutic methods. Frequently, courses in related fields such as human biology, physiology, cultural anthropology, and sociology are required. There is a highly academic insistence on developing skills in foreign language and on preparing a dissertation. Much greater emphasis is laid on preliminary, comprehensive, and oral examinations than in the schools of social work or psychiatry. Occasionally, clinical psychologists consider a psychoanalysis important in their education, but this is not emphasized as much as it is in social work or psychiatry.

The first observation we make in considering the training of psychiatrists is that there are several accepted routes to becoming a member of this profession. All have the first portion in common, which is the four years of medical school. In typical medical schools during these four years, usually less than one-twentieth of the time is devoted to clinical psychiatry, and occasionally a brief course in psychology is added. Certain skills and attitudes gained in practicing clinical medicine can be transferred to the practice of psychiatry and are of great importance. These include knowledge of the nature of illness; experience as to how human beings of various types react to their diseases; a comprehension of how to approach patients; an understanding of clinical method; an ability to bear responsibility for decision concerning their welfare—a sort of *esprit de corps* of medical integrity comparable to the morale inculcated into the cadets at West Point. Though they do obtain much relevant to psychiatric practice in medical school, it is a fact that, at present, medical students learn little direct psychiatry beyond an elementary understanding of diagnostic categories.

Three common roads lead to a career in psychiatry after medical school. The first includes a medical, neurological, or rotating internship together with further training in neurology. The primary interest of such doctors is in neurology and neuropathology, but, since they find that a large proportion of their practice is psychiatric, they are forced by practical considerations to call themselves neuropsychiatrists. Having had little psychiatric instruction, they usually take a “common sense” approach to such problems and rely heavily on advice, reassurance, vitamins, and trips to the country. With their “organic” neurological background they frequently express outspoken antipathy for any other type of psychiatry.

Second, there is the “state hospital” approach to psychiatry. After medical, rotating, or other types of internships, these physicians go to hospitals where most of the patients are psychotic. Their interest becomes largely focused on diagnosis, often in Kraepelinian terms, on legal questions, custodial care, and final disposal of the patient. It is the sober truth that, with the exception of shock treatment and a few other less widely employed procedures like prefrontal lobotomy, there is and has been no serious therapeutic attempt in most of these hospitals, largely because of the unresponsiveness of these illnesses to therapy and because the doctors have been so overworked in their other duties.

Third, there is the “dynamic” approach to psychiatry. This post-graduate training usually consists of a didactic analysis and then an apprenticeship under an analytically-minded psychiatrist—review of interviews, suggestions on how to conduct the psychotherapeutic course, and “control analyses.” In a few rare residencies only can the student get a broad selection of opinions and theories, including more than one man’s approach to psychotherapy or the doctrines of more than one school of psychiatry. Psychoanalytic institutes, however, conduct

courses and seminars in which the viewpoints of a number of instructors can be obtained, but the range of their attitudes is usually limited by their acceptance of a common basic doctrine. Rare is the inclusion of psychology, sociology, and anthropology in graduate psychiatric training.

Not only do the three professions of psychiatry, psychology, and psychiatric social work at present vary widely in their training, but also, largely because of these training differences, they have markedly divergent motivations. A point of importance about social workers is that most of them are women. This largely affects the professional motivation of the group, just as it does the attitudes of the nursing profession. Because they are women, they accept more easily working as submissive subordinates to the doctor and being constantly under the medical aegis. This makes for good social relations in the social worker-doctor team, but is not unequivocally good for the profession. When, during the war, the percentage of males acting as social workers became large in the Armed Forces, there was a marked increase in friction between the two professions. Physicians from time to time said they were distressed at the "new pretensions" of social workers, and the social workers for the first time pressed for a clear delineation of their duties, which before had not seemed of primary importance. Since most psychologists are male, it is likely that there will be more competition between them and psychiatrists than there has been in the past between social workers and psychiatrists.

- A motivational undercurrent of social work, which springs partly from the fact that a high percentage of the profession is feminine and partly from the fact that the profession is constantly in contact with official and semi-official social agencies, is a strong tendency to work toward maintaining the status quo of the social order. An important responsibility of social workers is to help order the environment in which the patient will live, and make disposition of the patient at the end of treatment in the way best calculated to do away with conflict. An important aspect of this is arranging it so that the patient lives in harmony with his society, and to do this the social worker is motivated to continue the recognized patterns of behavior in societies, whether or not he may intellectually wish to accomplish reform by community education or legislation. This is a markedly different approach from that of most psychiatrists or psychologists, who operate in private relationships which do not exert pressures toward conformity as the public contacts of the social worker do.

The largely masculine motivation of psychologists differs vastly from that of social workers. Among clinical psychologists, there is undoubtedly a greater idealistic urge to help suffering individuals than among any other group of psychologists, but even this idealism is commonly expressed by them in terms of the discovery of generalizations about human personality and mental disease which later is capable of application in the individual case. Psychology has dwelt most of its life amid

academic towers. It has long professed an idolatry of exact science, of predictable law, and of research to achieve these ends. The main stem of psychology has by no means felt itself to be a step-child of medicine, nor has it envied the role of the physician, except for his prestige in recent years. Rather, older psychologists have viewed clinicians haughtily as being beneath them because they are inexact impressionists. They have yearned to approach the precision of physiology, hoping some day to scale to the heights of accuracy of organic chemistry, physics, astronomy, and, above all, pure mathematics.

Even a superficial survey of the history of psychology reveals important facts about present motivation of psychologists. The metaphysical beginnings of psychology as a contemplative discipline were soon overcome by the development of a laboratory science patterned after physiology, begun by Wundt and Helmholtz in the middle of the last century. Until quite recently, this experimental approach has constituted the central stream of psychology. Emphasis has been placed on psychophysics, sensation and perception of external stimuli, learning, memory, attention, feeling, animal behavior, and similar fields in which precision of measurement has been possible. Studies of abnormal behavior, like those by Charcot, Prince, Janet, and Freud, have always contributed to psychological thought, but these have not received the main emphasis. Similarly, interest in group phenomena and the applications of psychological principles to masses in industrial and personnel psychology has been a sideline. In the last decade, there has been a tremendous increase in concern with psychoanalysis and all its offshoots, with the counseling techniques represented by Carl Rogers and others, and with the personalities and mental illnesses of individuals, so that during the war years there has been a real revolution, and the clinical psychologists, who were once underdogs because they dealt in fields where precision was difficult, have now become "top dogs," although the demand for precision and careful method still is loud and there are many different points of view in psychology. This divergence in viewpoint among psychologists is diluted by the extremely broad range of matters with which they concern themselves, a scope far greater than the treatment of ill or socially maladjusted individuals, which is the primary concern of psychiatrists and social workers. Today, psychologists are writing advertisements, choosing personnel, counseling normal individuals on vocational and marital matters, diagnosing and treating mentally ill patients, studying characteristics of human sensations, investigating public opinion, gauging social trends, determining principles of proper education, recommending to engineers how to arrange instrument panels, designing houses to suit human limitations and sentiments, analyzing administrative organizations, and devising methods for preventing fatigue and safety hazards, as well as carrying out many other diversified activities.

The professional purpose of psychologists, then, clearly differs from the aims of the other professions, though the recent revolution has

brought it closer rather than farther away. It differs from the well-defined goal of the medical psychiatrist and social worker, which is to help the human being who needs aid now. The psychologist characteristically hopes to determine principles of normal and abnormal behavior, with emphasis on the normal, as precisely as possible, with the expectation that once these laws have been determined they can be applied for the welfare of men.

Distribution of Duties Forced by Present Circumstances. We can now consider the training situation in clinical psychology. What sort of education in this field is it necessary and feasible to ask a university to give at the present time?

Let us hope, that, by now, most trade-unionism can be dispersed; that professional antagonisms will not be so bitter as to be determining factors; that legal restrictions can be surmounted or circumvented; and that assignment of diagnosis, therapy, research, and other clinical activities can be to the best qualified person, whoever he may be. Let us, therefore, neglect such possible hindrances in our present considerations.

It is impossible, however, to sidestep the tremendous demand which exists and which will continue to exist indefinitely for services in clinical psychology. The requirements of the Armed Services in the last war found the country to be shorter of psychiatric service than of any other medical specialty. If the country is to continue adequate mental hygiene and hospitalization programs in psychiatry, the supply of psychiatrists will be far short for many years. Group therapy alone does not give promise of settling the problems of how psychotherapy will be given to the masses who need it. Inevitably, whether we want it or not, psychiatric social workers and clinical psychologists will be called upon to assume therapeutic roles.

A report on an official Army survey of duties performed by clinical psychologists in the Army during the last war indicates that, from January 1945 to April 1946, about one quarter of the time of psychologists was spent in doing guidance and therapy. The authors of the report state:*

"Most of the time devoted [by clinical psychologists] to therapy was for individual therapy; the rest of the time was given to counseling and to group psychotherapy. It is significant that these therapeutic efforts were carried on under the direct supervision of the neuropsychiatrist. While, in part, this function represented the desires of psychologists to participate in this work, in much larger measure it was the result of a tremendous patient load in neuropsychiatric and in neurological sections which could not be handled by neuropsychiatrists alone, and so involved both clinical psychologists and psychiatric social workers. As psychologists gained experience along this line, they were used more frequently to meet the emergency needs. The functions of re-education car-

* HURT, M. L., & E. O. MILRON. The duties performed by clinical psychologists in Army Medical installations. *Bull. Mil. Clin. Psychol.* 1: 115, 117. 1946.

ried by psychologists with aphasic patients and with other physically handicapped patients (such as the paraplegics and the deafened) contributed to the relatively large amount of time devoted to guidance and therapy.

"Many of the narrative comments supplied by psychologists indicated that they were more interested in careful diagnostic work and particularly in clinical research in psychodynamics and psychotherapy than in therapy as such. In these areas . . . the psychologist, basing his service on solid training and scientific method, has a far more important contribution to make [than in psychotherapy], especially under less pressured situations."

These last statements undoubtedly represent the majority opinion of qualified clinical psychologists. In general, they believe that their most important contributions can be in fields other than direct therapy, and therefore they hope to evade extensive involvement in psychotherapy. It must be admitted that there are a number of clinical psychologists whose chief purpose in entering the field, like that of some psychiatric social workers, is in order to do therapy—but this is a minority, and the developmental history of psychology makes it clear why this is so. Anyone following non-medical roads to therapy merely because they are shortcuts (though clinical psychology is not a very short shortcut) of course deserves disapproval.

Is it not reasonable to ask here, however, whether a graduate clinical psychologist or psychiatric social worker is not better trained to do "dynamic" psychotherapy than the "psychiatrist" who has chiefly a neurological background, the "psychiatrist" with a custodial, state hospital background, or the general practitioner? Some will argue that the skills transferred from general medicine to such clinical work are so important that any physician is better trained for such therapy than any layman. One bit of evidence advanced to support this position is that the young medical officers in the Army just out of their internships who were given ninety days of psychiatric training were thought by some to make, in general, better therapists than experienced clinical psychologists. If this judgment is correct, it still would be difficult to tell whether this is the result of transfer of general medical training or the consequence of the fact that the prestige of medicine in our society and the excellence of medical aptitude tests together serve to select for medical training young men of outstanding all-round ability who therefore are capable of superior performance in any task they undertake.

The demand for psychiatric treatment is so great that ten times as many psychiatrists as we now have probably could not handle it adequately. Therefore, the doctor must decide which parts of the therapy in this field he wishes to handle himself. Does he wish to do occupational therapy, recreational therapy, habit retraining, re-education of aphasics, other speech therapy, counseling on normal adjustment problems, or vocational counseling? Almost all of these therapies are already recognized

to be the province of psychologists or other technicians, and very little complaint is heard from physicians. There can be no doubt that these are therapies in a respectable sense of the word.

Even when the psychiatrist delegates all these functions, he still has an enormous task in the direct treatment of the more severe mental maladjustments. The jokes about patients growing long white beards waiting to be analyzed and the comments about only the rich being able to afford neuroses are not humor untouched with reality. If America is going to make a serious effort in the next decade to give therapy for mental maladjustments where it is needed, large numbers of psychotherapists must be trained—more than medical schools with their present limited enrollments will be able to turn out. There are three possible sources: multiply medical school enrollment at least by two; train lay psychotherapists; or evade the responsibility to give help where needed, as, for instance, we have neglected the scourge of pellagra in certain sections of the South. The first course seems highly improbable, at the moment, and the last would have many serious consequences, not the least of which would be the turning of the ill to the psychological cultists.

Even if this problem were not so critical, the reasons mentioned above as to why a psychologist should do therapy to improve his diagnostic and research skills are cogent enough. The conclusion, therefore, must be that for both idealistic and realistic reasons the ideal university will train clinical psychologists in coming years to carry out the following three functions: diagnosis, research, and therapy.

A Specific Training Program. In planning education in the various psychological professions, we must accept the principle that each of the three chief professions involved should have different training, which follows directly from our earlier decision that there should be specialization.

We shall not go into detail as to what should be required for social workers, except to say that, if they are to do case work such as they have done in the past, which often means carrying out extensive therapy, they should receive longer and more intensive formal training than they have in the past. This preparation should be based on the work they are to do, and if their responsibilities are to include therapy, they should have the necessary broad background for this, as well as supervised practice (in which social work has been stronger than either psychology or psychiatry). It is not fair to the patient to have him treated by unskilled therapists, using as a rationalization the semantic distinction between case work and therapy.

In the training of psychologists and psychiatrists, there should be as much merging of backgrounds as possible. It would be well for modern universities to develop graduate schools with curricula permitting a number of individuals who wish to have really thorough preparation to become qualified in both psychiatry and clinical psychology. A possible

schedule might be something like the following: two years of liberal arts college; one year of advanced clinical psychology, sociology, and cultural anthropology; one year of the pre-clinical medical sciences which would be comparable to the present first year of medical school. At the end of these four years, a bachelor's degree would be granted, following which the candidate would take the second and third years of medical school work and then spend a year in psychiatric clinical work in a general hospital, in a mental hygiene clinic, or in a neuropsychiatric hospital. At the end of this time, he would be granted the M.D. degree in psychological sciences. He then would have one year of a rotating psychological-psychiatric internship, which would include both psychological examinations of all sorts and the performance of various psychiatric duties, complementary functions of equal status. This would be followed by one year of independent research leading to a dissertation, seminars, and perhaps a psychoanalysis. At the end of this year, a doctoral degree in clinical psychology might well be awarded. Work from this time until the candidate is qualified for his specialty boards in psychiatry or clinical psychology might consist of mixed psychological-psychiatric residencies, including work with psychoneurotics, psychotics, psychosomatic patients, and clinical research.

There would necessarily be other individuals, and in larger numbers, who would want either a psychiatric education alone or a psychological education alone. The same graduate school that set up the combined program outlined above should be able to give both these latter courses of training, because even if the curricula are separate they should be closely integrated, since clinical psychologists and psychiatrists should learn from the beginning of their training to operate as a team.

The report on graduate training in clinical psychology made by the Subcommittee on Graduate Internship Training of the American Psychological Association and the American Association of Applied Psychology outlines the most satisfactory plan yet suggested.* It begins by suggesting that the undergraduate program of students in this field should include 20 semester hours in psychology, to consist essentially of the standard courses for undergraduates, not including professional or advanced work—courses like beginning psychology, beginning abnormal psychology, psychological statistics, learning and perception, the higher mental processes, physiological psychology, etc.; 20 semester hours in biology, physics, and chemistry; 9 semester hours in mathematics, through introductory calculus and statistics; 9 semester hours in the fundamentals of educational philosophy and experimental didactics; 12 semester hours in anthropology, sociology, economics, and political science; 6 semester hours in the history of culture, philosophy, logic, and comparative literature; and enough work in modern languages to develop a reading knowledge of two, preferably French and German.

* Subcommittee on Graduate Internship Training to the Committee on Graduate and Professional Training of the American Psychological Association and the American Association for Applied Psychology (D. SHAKOW, Chairman). *J. Consult. Psychol.* 9: 243-266. 1945.

Building on this general background, the report suggests a four-year graduate program leading to the doctorate, including a full year of internship, preferably the third year. The courses recommended for the first year are general psychology, 6 semester hours; dynamic psychology I, 3 semester hours; experimental clinical and dynamic psychology, 3 semester hours; developmental psychology, 6 semester hours; theory and practice of psychological tests and measurements I, 3 semester hours; physiological sciences, including physiology, anatomy, neuroanatomy, neurophysiology, and endocrinology, 6 semester hours; advanced statistics and qualitative methods, 3 semester hours. In the second year, the committee recommends dynamic psychology II, 3 semester hours; experimental clinical and dynamic psychology II, 3 semester hours; theory and practice of psychological tests and measurements, 6 semester hours; theory and practice of projective devices, 3 semester hours; therapeutic theory and methods, 6 semester hours; methods of case study and analysis, 3 semester hours; introduction to clinical medicine, 3 semester hours; educational and vocational guidance technique, 3 semester hours. The third year consists of an internship with various sorts of psychiatric patients. The recommendations for the fourth year include independent research leading to a dissertation; cross-discipline seminars attended by representatives of psychology, anthropology, sociology, psychiatry, etc.; seminars in professional problems, standards, ethics, etc., of psychology; and additional courses in psychology as needed to round out the individual's curriculum.

The new thinking contained in this report is evidenced in various ways: by inclusion of pre-clinical sciences; by emphasis upon training in medicine, neuroanatomy, and neurophysiology; by emphasis upon related fields like sociology and anthropology; and above all by stressing the clinical rather than the academic approach, following medicine in teaching as much as possible at the patient's bedside. It would not, however, constitute adequate preparation for independent psychotherapy, which would have to be obtained later if required.

The most satisfactory way to give such a psychological curriculum might well be in a separate graduate school for the psychological sciences. Here, certain basic graduate courses could be given, and then specialization could be undertaken in medical or clinical psychology, in pure research and experimental psychology, in social psychology, in industrial psychology, or in other areas. There should be close liaison with the medical school, the law school and the business school, and combined programs with all of them.

Psychiatric education needs as radical alterations of the traditional program as does psychological education. The changes should begin in undergraduate medical school. First, an inclusive and well-planned course in psychology, including laboratory work, should be presented in the first year in the same status as the other pre-clinical sciences of normal function, like biochemistry and physiology. Second, there should

also be, to a lesser degree, opportunity for study in sociology and economics. Third, since at least half the cases seen by a general practitioner have significant psychiatric aspects, certainly psychiatry should be taught in every one of the clinical years and should include much more emphasis on psychoneurosis and psychosomatic medicine than on psychosis. Fourth, there should be a real course in applied clinical psychology, including psychometric testing, the use of projective techniques, and all other methods available to clinical psychologists. Medical students should have an opportunity to use these procedures in order to familiarize themselves with the theory behind them and to understand the significance of reports based on their use. Fifth, there should be courses in biostatistics and in the scientific method. Sixth, medical students should be required to do independent investigation, resulting in a dissertation in a field of their choice, so that they will have respect for, motivation for, and understanding of the importance of research. Last, medical students should have the opportunity to work as members of the neuropsychiatric team.

After medical school, interns and residents in psychiatry should learn more than how to make diagnoses and give shock treatment. There should be instruction in psychoanalytic and other dynamic psychiatric concepts. A valuable adjunct to this is the study of literature, art, and history from this dynamic viewpoint. There should be instruction in psychotherapy both in an apprenticeship under an individual instructor, and in groups making detailed studies of the therapeutic progress of individual cases under the direction of various psychiatrists, so that different approaches can be learned. Student psychiatrists should become proficient in organizing and participating in the neuropsychiatric team. Finally, emphasis upon clinical research should be markedly increased in psychiatry, because of the great need for advancement in this field.

It is clear that all the professions in the psychological field will in the future be working together. Thousands need to be trained in each of them. The educational programs must be united in a pattern as closely integrated as the cooperative activities in which their graduates will take part.

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